Ontogeny and Pharmacogenetics:

Determinants of Age-Associated Differences in Drug Clearance

During Human Development

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Ontogenese en farmacogenetica:

Determinanten van leeftijdgebonden verschillen in klaring van geneesmiddelen

tijdens de ontwikkeling van de mens

Proefschrift

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Chapter 1

Introduction

1.1 The "Therapeutic Orphan": A Demand for Diligence and New Direction in Pediatric Clinical Pharmacology

The 1962 Kefauver-Harris amendments to the United States Federal Food Drug and Cosmetic Act provide that, in addition to being safe, a new drug must be shown by substantial evidence to have the effect it is purported to have under the conditions of use prescribed, recommended, or suggested in its labeling before it may be approved for human use. However, an unwitting and unfortunate consequence of the 1962 amendments for infants and children has been the introduction of exclusionary wording into the labeling of most drugs that disclaims use of the drug for pediatric patients. Studies to support labeling for children had not been done for the majority of drugs over the past 3 decades and, consequently, children have been systematically excluded from the intended benefits and safeguards of this 1962 Act.

Following promulgation of the 1962 amendments, Dr. Harry Shirkey, coined the term "therapeutic orphan" to decry the exclusion of children from the drug approval process and the dearth of scientific knowledge used to support the provision of individualized drug treatment for pediatric patients.[1] The plight of the "therapeutic orphan" was highlighted seven years later by Wilson[2] who reviewed the 1973 issue of the Physician's Desk Reference and demonstrated that 62 % of all listed approved drugs in the U.S. did not include dosage recommendations for children and furthermore, that 78 % of the monographs disclaimed use of a given drug in children or failed to supply age-specific dosing guidelines.

During the ensuing years, new technologies in analytical chemistry, pharmacokinetics and pharmacodynamics facilitated the study of drug disposition and action in pediatric patients and as a result, enhanced therapeutic drug use in infants and children. Concurrently, there have been multiple international and national efforts to address the ethical and regulatory restrictions that had previously hampered pediatric clinical pharmacology research. Collectively, the results of these efforts exerted over nearly a quarter century are both encouraging and disheartening. Knowledge of the impact of development on drug disposition and, to a lesser extent, drug action vastly improved in both amount and scope,[3] thereby enhancing the science associated with pediatric therapeutics. In stark contrast, this "new knowledge" was not effectively translated to

prescribers, parents and/or patients through the tested and time-honored process of drug development in the U.S. and Europe; namely, by placing pediatric clinical pharmacology information (e.g., pharmacokinetics, dose selection, adverse event profiles, etc.) into the approved product labeling. This incongruity of purpose and outcome was demonstrated by a survey of the 1991 Physician's Desk Reference which demonstrated that 62 % of monographs (i.e., labels) for prescription drug products approved and marketed in the U.S. were either devoid of pediatric dosing recommendations or alternatively, provided exclusionary language regarding the use of a given drug in infants and/or children.[3] As well, a 1988 survey of 80 hospitals that treated pediatric patients conducted by the U.S. Food and Drug Administration demonstrated that 70 % of the drugs routinely used in the treatment of infants lacked a pediatric evaluation section in the product labeling.[4] A review of new molecular entities, which represent the most innovative new medications, approved by the FDA from 1984 through 1994 showed that 80 % were approved without labeling for children, although many are widely used to treat children.[5] Finally, the pediatric "information deficit" for proprietary medications is not solely a U.S. phenomenon but rather, one of global proportions as evidenced by recent studies of off-label pediatric drug use in Germany[6] and the Netherlands.[7] Thus, pediatric patients represent a uniquely disenfranchised sub-population of society where "off-label" drug use (and its attendant risks) predominates and therapeutic drug use is frequently not guided by data from appropriately designed, adequate and well controlled clinical trials as is required for adults.

After more than four decades of virtually absent pediatric drug regulation, leaders in the field of Pediatric Clinical Pharmacology were successful in using the evidence of their scientific labors to convince politicians and society to enact regulations (e.g., United States Food and Drug Modernization Act of 1997; United States Best Pharmaceuticals for Children Act of 2001)[8,9] and guidance documents (e.g., ICH E-11 Guidance) to foster the inclusion of infants, children and adolescents as subjects in clinical trials of drugs that had the potential to favorably impact pediatric therapeutics. As a direct result, the past decade has seen a true renaissance in the field of Pediatric Clinical Pharmacology. This is evidenced by the investigation of more than 50 drugs in over 32,000 pediatric patients conducted by global pharmaceutical companies in the U.S. and Europe from January of 1998 through March of 2002; a more than 500 % increase in the number of pharmaceutical company sponsored pediatric clinical trials conducted in the preceding 10

years. This dramatic increase in pediatric clinical pharmacology research has not only expanded the base of scientific knowledge but also, has been translated into information required to rescue the "therapeutic orphan". Specifically, in the United States, pediatric clinical trials conducted as a direct result of the pediatric provisions of the Food and Drug Administration Modernization Act of 1997[8] have resulted in revision of the approved product labeling of over 30 drugs to specifically add pediatric information for agents used to treat a variety of conditions commonly encountered in pediatric patients (e.g., cardiac arrhythmias, diabetes, gastroesophageal reflux, hypertension, HIV infection, juvenile rheumatoid arthritis, obsessive compulsive disorder, depression, pain, epilepsy). As a direct result, children of the World are benefiting from pediatric-targeted drug development and discovery in real time.

While the focal point of these new efforts in Pediatric Clinical Pharmacology has been (and rightfully so) improved safety and efficacy of drugs for infants, children and adolescents, another major benefit goes somewhat unrecognized; namely, the integration of translational science into study design and conduct and the power of these experimental tools to provide new discoveries about normal human development. As is illustrated by Chapter 2 of this thesis, excellent investigations performed in the past clearly illustrate that human ontogeny exerts profound effects upon the disposition (i.e., pharmacokinetics) and action (i.e., pharmacodynamics) of drugs; differences that translate into the need for age-dependent approaches for the provision of safe and effective drug therapy. These data provide a stark reminder that "children are not simply small adults" and most importantly, they provide the revelation that developmental differences truly exist and are, in most cases, of profound clinical significance. As has been previously demonstrated, pharmacokinetic data for many drugs can provide a "road map" for development by revealing an age-specific "pattern" reflective of change and/or reveal those periods of life where the most dramatic differences occur. While the arguments and indications from such data are often quite compelling with respect to their potential scientific and/or clinical significance, there are still critical "missing links". Namely, what are the independent and/or collective factors that produce developmental differences in drug disposition (e.g., drug metabolism) and when developmental differences in pharmacokinetics are observed, how much of the difference is attributed to development *per se* vs. normal interindividual variability in the processes that collectively, are the determinants of drug disposition?

The following thesis is intended to not merely represent a treatise on Pediatric Clinical Pharmacology or to demonstrate its feasibility and importance but most importantly, to illustrate a process of discovery through the marriage of old (i.e., pharmacokinetics) and new (i.e., pharmacogenetics) scientific approaches integrated into clinical investigations of drugs important to pediatric patients. The overall purpose of this work is to provide examples of how such integration is essential in revealing the true impact of ontogeny on drug disposition. As such, the goals of the research described in this thesis were:

1. To review the current knowledge of the impact of ontogeny on drug disposition (Chapter 2) and to provide an example using a previous pharmacokinetic study of a drug used extensively in newborn infants (i.e., metoclopramide) that illustrates how dynamic developmental differences and interindividual variability impact upon drug dose – exposure relationships (Chapter 3).

2. To demonstrate how development can dramatically influence the pharmacokinetics of a drug (e.g., linezolid) that is not metabolized by hepatic microsomal enzymes but rather, is catabolized by ubiquitously expressed enzymes which, as revealed by pharmacokinetic studies, have a clear developmental pattern for their expression (Chapters 4 and 5).

3. To review the historical information that supports a profound impact of ontogeny on the expression of the most abundant of the human cytochromes P450 (i.e., CYP3A; Chapter 6) and to illustrate how development influences the expression of CYP3A by providing data from pharmacokinetic studies of a pharmacologic substrate for this enzyme (midazolam) in infants, children, adolescents (Chapter 7) and neonates (Chapter 8).

4. To illustrate the development of a selective pharmacologic probe for CYP3A4 (i.e., cisapride) suitable for pediatric use (Chapters 9 and 10) and to demonstrate proof-of-concept for this probe as a means to examine the ontogeny of this cytochrome P450 isoform in neonates and young infants (Chapter 11).

5. To demonstrate the new frontier for developmental pharmacology studies by reviewing the challenges of integrating relevant pharmacogenetics and pharmacogenomics into pediatric drug development and clinical therapeutics (Chapter 12) and providing two contrasting examples where the integration of pharmacogenetics into a pediatric pharmacokinetic study provided both confirmatory information (Chapter 13) and a new discovery (Chapter 14) relative to polymorphically expressed drug metabolizing enzymes.

1.2 References

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Chapter 2

Developmental Pharmacology: The Impact of Ontogeny on Drug Disposition and Action

Based on the article:

Developmental Pharmacology: The Impact of Ontogeny on Drug Disposition and Action

Kearns GL, Abdel-Rahman SM, Alander SW, Blowey DL, Leeder JS, Kauffman RE.

2.1 Introduction

From a societal, psychosocial, behavioral and medical perspective, infants and children are far different from adults. This is certainly true in the area of pediatric therapeutics where the impact of ontogeny on the processes that collectively determine drug disposition and action require that drug treatment (i.e., selection of both drug and dosing regimen) be appropriately tailored for age in order to insure safety and efficacy. To accomplish this objective, it is essential that prescribers have working knowledge of how development can influence the pharmacokinetic-pharmacodynamic interface.

2.2 Materials and Methods

The Pediatric Patient

More than 100 years ago Dr. Abraham Jacobi, the father of American Pediatrics, stated: "pediatrics does not deal with miniature men and women, with reduced doses and the same class of disease in smaller bodies, but...it has its own independent range and horizon..."[1] During the past century, tremendous strides have been made to tailor therapies for the needs of children. As our knowledge of normal growth and development has increased, so has our recognition that developmental changes profoundly affect the responses to medications and produce a need for age-dependent dose requirements.

Over the past 30 years, numerous approaches (e.g., Young's Rule, Clark's Rule) for determining pediatric drug doses have been recommended in an attempt to accommodate developmental differences. These approaches vary, some using discrete age points and others using allometric principles that generally assume predictable, linear relationships between mass (e.g., cell mass, body weight) and/or body surface area between infants, children, adolescents and adults.[2] However, as human growth is not a linear process and age-associated changes in body composition and acquisition of organ function are dynamic and in some instances, discordant during the first decade of life, none of these approaches are adequate for individualizing dosing regimens across the span of childhood.[3] As a result, these "dosing equations" have been abandoned and the usual practice for pediatric drug dosing remains one of simple "normalization" of dose as a function of either body weight (mg/kg) or body surface area (mg/m^2) as the means of translating doses between children of different ages. While this approach is generally

adequate for assignment of individual dose, it does not permit dose scaling between age groups where dramatic developmental differences in the normalized dose (i.e., mg/kg or mg/m^2) and/or required dosing interval may occur. To provide safe and effective drug therapy for pediatric patients, it is necessary to integrate the complex changes that accompany normal growth and development with their impact on the ontogeny of drug disposition and action.

Absorption

For drugs administered by extravascular routes, several barriers to drug entry exist and, it is the ability of a drug to overcome these chemical, physical, mechanical and biological barriers that defines the process of absorption. Developmental differences in the physiologic composition and function of these barriers can, for many drugs, manifest as alterations in the rate and/or extent of absorption in children.

Although drug is delivered directly to the site of absorption for most extravascular routes, this is not so for the principal route of administration, oral drug delivery. In addition to physiological barriers, developmental changes in the perception of taste, texture and odor represent additional potential limiting factors for the retention of orally administered drugs.[4] The ability to perceive sweet tastes appears within days of birth whereas neonates and infants prove somewhat indifferent to salty, sour and bitter tastes. An adult pattern of salt preference and an aversion to sour and bitter tastes is fully refined by approximately two years of age. Similarly, the ability to detect odor is present soon after birth; however, children do not appear to reliably discriminate between pleasant and unpleasant odors until 5 to 7 years of age.[4] Thus, adult perceptions of taste and smell are not reliable predictors of drug acceptability to the preschool aged child where a drug formulation with an unpleasant taste or texture that is masked by a pleasant odor may likely not be acceptable.

Developmental changes in the gastrointestinal tract can also alter the extent of drug absorption. Changes in luminal pH directly impact both drug stability and degree of ionization, thus influencing the relative amount of drug available for absorption. During the neonatal period, intragastric pH is elevated (e.g., > 4) consequent to reductions in both basal acid output and total volume of gastric secretions.[5,6] Thus, greater oral bioavailability is seen for acid labile compounds (e.g., penicillin G) in neonates when

compared to older infants and children.[7] In contrast, weak acids (e.g., phenobarbital) may require larger oral doses in the very young to achieve therapeutic plasma concentrations.[8] Additionally, the ability to solubilize and subsequently absorb lipophilic drugs or formulations can be influenced by age-dependent changes in biliary function. Immature conjugation and/or transport of bile salts into the intestinal lumen results in low intraduodenal levels despite blood levels that exceed those of adults.[9,10] Pleconaril, an antipicornaviral, demonstrates dose proportionality in adults. However, when formulated in a lipid-based vehicle, apparent capacity limited absorption results in neonates which may be a result of inadequate intestinal bile salt concentrations necessary to sufficiently solubilize the drug.[11]

Development can similarly influence the rate of drug absorption. Gastric emptying rate and intestinal motility are primary determinants of the rate at which drugs are presented to and dispersed along the mucosal surface of the small intestine. At birth, the coordination of antral contractions improves and the rate of gastric emptying increases markedly during the first week of life.[12,13] Similarly, intestinal motor activity matures through early infancy with the frequency, amplitude and duration of propagating contractions increasing.[14] Few studies have systematically evaluated the effect of these developmental changes on drug absorption in a large number of children over a broad range of ages. However, the absorption of nutrient macromolecues subject to both passive (e.g. arabinose) and active (e.g. xylose) transport suggest acquisition of full function by approximately 120 days of age.[15] Similar observations have been observed in small numbers of infants following the oral administration of phenobarbital, sulfonamides and digoxin.[15] As such, the rate at which most drugs are absorbed is generally slower and the time to achieve maximum plasma concentrations prolonged in neonates and young infants.

Developmental factors in addition to gastrointestinal transit time play a role in limiting absorption rates in the neonate and young infant. While it is generally assumed that reduced intestinal surface area exists early in life, average intestinal length as a percentage of the adult value exceeds other anthropometric measurements throughout development.[16] Given that villous formation begins at eight weeks of fetal life and matures by week 20,[17] it is unlikely that reductions in small intestinal surface area contribute to reduced absorption. However, age-associated changes in splanchnic blood flow in the first 2-3

weeks of life[18-20] may influence absorption rates by altering the concentration gradient established across the intestinal mucosa.

Despite their incomplete characterization,[21] developmental differences in the activity of intestinal drug metabolizing enzymes and active efflux transporters in the small intestine have the potential to markedly impact oral drug bioavailability. Biopsy specimens from infants and children suggest that epoxide hydrolase and glutathione peroxidase activities demonstrate little age dependence, whereas intestinal CYP1A1 activity appears to increase with age.[22] In contrast, distal duodenal biopsies suggest a reduction in glutathione-S-transferase activity with age, the impact of which can be observed on the apparent oral clearance of busulfan.[23] Although data on developmental expression of P-glycoprotein in humans is absent, the activity of other carrier-mediated active uptake processes (e.g. metal transporters) in the intestine clearly increases with age.[24] Finally, changes in the composition of intestinal microflora during infancy may contribute to the pre-systemic metabolism for a number of drugs as evidenced by age dependence in urinary excretion of digoxin reduction products.[25]

Development also impacts drug absorption by other extravascular routes. Enhanced percutaneous absorption during childhood is accounted for by a number of factors. A thinner stratum corneum plays a role in the neonate but by 26 weeks, it is similar to that of a term infant.[26] Also, the greatest differences in skin hydration are observed in the premature infant, although hydration remains greater than observed in adults throughout childhood.[27,28] Cutaneous perfusion is also markedly increased in young children as compared to adults.[28] As well, the ratio of total body surface area to body mass in infants and young children far exceeds that of adults. Thus, relative systemic exposure to drugs applied topically to infants and young children (e.g. corticosteroids, antihistamines, antiseptics, insect repellants) may exceed that in adults and produce the potential for toxicity.[29,30]

Reduced skeletal muscle blood flow and inefficient muscular contractions (responsible for drug dispersion) may reduce the rate of intramuscular drug absorption in neonates.[31] However, the impact of these factors may be offset by the increase in skeletal muscle capillary density observed in infants as compared to older children.[32] Accordingly, evidence supports more efficient intramuscular drug absorption and higher peak plasma

concentrations for a number of agents (e.g. antibiotics, anticonvulsants) in neonates and infants as compared to adults.[33,34]

Rectal drug absorption is also influenced by development. Rectal bioavailability for several agents is enhanced in the young consequent to reduced metabolism rather than enhanced mucosal translocation. However, enhanced lower gastrointestinal motility, reflected by a greater number of high amplitude pulsatile contractions can enhance rectal expulsion of solid dosage forms[35] and thereby, reduce drug absorption (e.g. erythromycin, acetaminophen).[36-38] In addition, physicochemical properties of the drug and/or formulation (melting temperature, particle size, drug-vehicle interactions)[39] may impact rectal drug absorption in pediatric patients.

Drug delivery to the lung is increasingly being used in pediatrics. While the principal target of these therapies is to achieve local effect, systemic exposure occurs as is evidenced by cortisol suppression after inhaled corticosteroid therapy.[40] While developmental changes in the architecture of the lung and ventilatory parameters (e.g., minute ventilation, lung capacity, respiratory rate) likely produce changes in drug deposition and absorption following intra-pulmonary delivery, current investigations restrict their evaluation to the effects that either the device or the formulation impart on application of the drug to (e.g. sedimentation, impaction, deposition) rather than absorption from the lung.[41]

Distribution

Age-dependent changes in body composition (Figure 1)[42] alter the physiologic "spaces" into which a drug may distribute and in many cases, the apparent volume of distribution. Larger extracellular and total body water spaces in neonates and young infants, coupled with adipose stores that have a higher water/lipid ratio than in adults, produce lower plasma concentrations for drugs that distribute into these respective compartments when administered in a weight-based fashion (Figure 2).[43] For lipophilic drugs that associate primarily with tissue, the influence of age on the apparent volume of distribution for most drugs is not as readily apparent. Changes in the composition and amount of circulating plasma proteins can also influence the distribution of highly bound drugs. A reduction in the quantity of total plasma proteins (including albumin) in the neonate and young infant results in a higher free fraction of drug.[44,45]

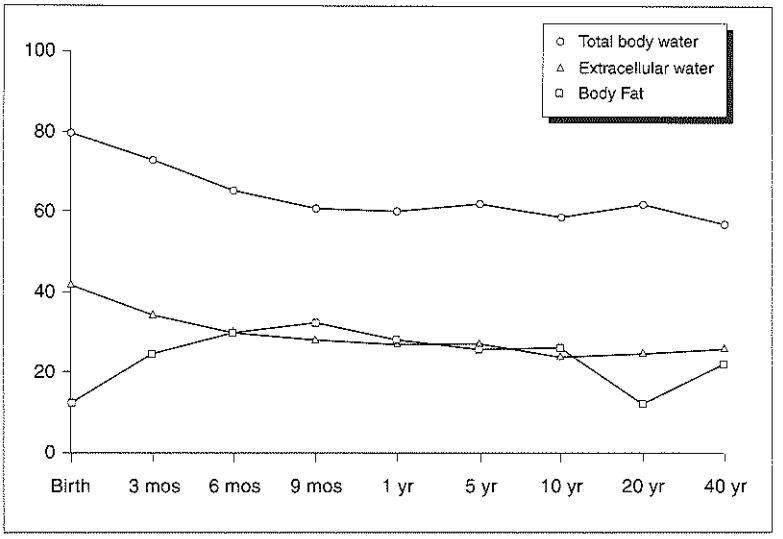

Figure 1 *Changes occurring in body fat and water stores along the continuum of age expressed as a percentage of body weight (adapted from data in reference 42).*

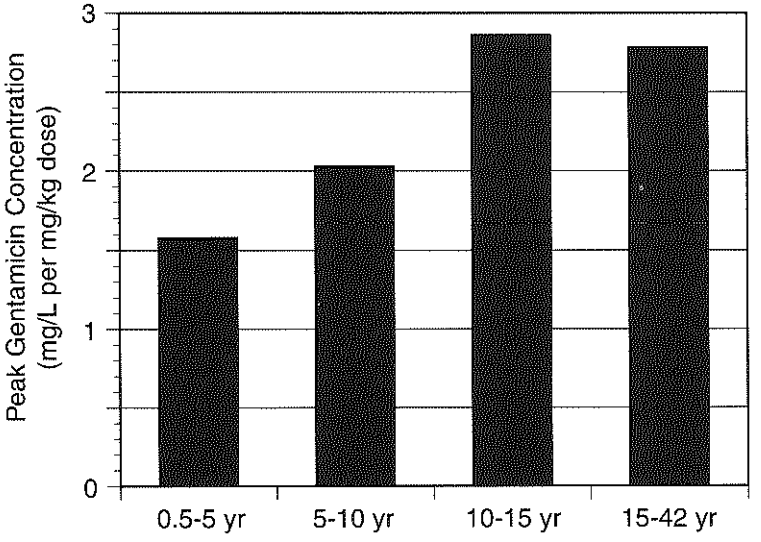

Figure 2. *Resultant gentamicin peak plasma concentrations following administration of the same weight-based dose to infants, children, adolescents and adults (adapted from data in reference 43).*

The presence of fetal albumin (which has reduced binding affinity for weak acids) and an increase in endogenous substances capable of displacing drug from binding proteins (e.g., bilirubin and free fatty acids) also contribute to higher free fractions of highly protein bound drugs,[44-46] a phenomenon largely limited to the first month of life. Other factors such as regional blood flow, organ perfusion pressure, permeability of cell membranes, changes in acid-base balance and cardiac output can also influence the rate and extent of drug distribution.

Limited data exist on organ specific drug distribution patterns during development. However, when drug entry into a given organ is governed by unique mechanism, as in the central nervous system, the available data merit discussion. To date, a single study of P-glycoprotein in infants 23-42 weeks gestation suggests that expression in the CNS increases during gestation and by term, demonstrates a localization pattern comparable to that of adults. However, the level of protein expression appears to be reduced overall as compared to adults.[47] Additionally, animal studies suggest that changes in blood flow and pore density (rather that pore size) are responsible for an increase in diffusion coefficients observed in neonates and young infants. However, these data have not been systematically evaluated in children and limited neonatal data suggest that brain to plasma ratios of phenobarbital concentration actually increase between 28-39 weeks gestational age.[48]

Drug Metabolism

Cardiovascular collapse associated with the "gray baby syndrome" in newborns treated with chloramphenicol is often cited as a clinically significant consequence of developmental deficiencies in drug metabolizing enzyme activities.[49,50] Multiple examples exist of developmental changes in drug biotransformation sufficient to have clinical import with regard to dose regimen selection in neonates and young infants (e.g., methylxanthines, nafcillin, 3rd generation cephalosporins, captopril, morphine). Current data reveal that distinct patterns of isoform-specific developmental changes in drug biotransformation are apparent for many Phase I (primarily oxidation) and Phase II (conjugation) drug metabolizing enzymes.

Development *per se* has a profound effect on the expression of Phase I enzymes such as the cytochromes P450 (CYPs). CYP3A7 is the predominant CYP isoform expressed in

fetal liver where it may play a fetoprotective role by detoxifying dehydroepiandrosterone sulfate[51] and potentially, teratogenic retinoic acid derivatives.[52] CYP3A7 expression peaks shortly after birth and then declines rapidly to levels that are undetectable in most adults.[53] Postnatally, distinct patterns of isoform-specific developmental CYP expression have been observed *in vitro*. Within hours of birth, CYP2E1 activity surges[54] followed closely by the onset of CYP2D6 expression.[55] CYP3A4 and CYP2C (CYP2C9 and 2C19) activities appear during the first week of life[53,56] whereas CYP1A2 is the last hepatic CYP to be acquired with significant expression being delayed until 1-3 months of life.[57]

Insight into the ontogeny of drug metabolism also can be derived from pharmacokinetic studies of drugs primarily metabolized by a single CYP isoform. When administered intravenously, midazolam clearance reflects hepatic CYP3A activity[58] which increases approximately 5-fold (1.2 to 9 ml/min/kg) over the first 3 months of life.[59] Carbamazepine plasma clearance, also largely dependent upon CYP3A4 activity,[60] has been reported to be greater in children relative to adults,[61,62] thereby necessitating higher therapeutic doses of the drug on a body weight (i.e., mg/kg) basis.

CYP2C9 and to a lesser extent, CYP2C19, are primarily responsible for phenytoin biotransformation.[63] Phenytoin apparent half life is prolonged (~ 75 hr) in preterm infants but decreases to ~ 20 hr in term infants less than one week postnatal age and to ~ 8 hr after two weeks of age.[64] Saturable phenytoin metabolism, a hallmark of CYP2C9 activity,[63] does not appear until approximately 10 days postnatal age, demonstrating the developmental delay in CYP2C9 activity.[65] Furthermore, pharmacokinetic data from children 0.5 to 15 years of age reveal that mean values for Vmax (reflective of CYP2C9 activity) decline from 14 mg/kg/d in infancy to 8 mg/kg/d in adolescence,[66] producing a profound corresponding difference in daily therapeutic dose requirement.

Caffeine and theophylline are the most common CYP1A2 substrates used in pediatrics. Caffeine elimination *in vivo* mirrors that observed *in vitro* with full 3-demethylation activity (mediated by CYP1A2) observed by four months of age[67] and somewhat later in breast-fed infants.[68] Formation of CYP1A2-dependent theophylline metabolites reaches adult levels by approximately 4-5 months of postnatal age[69] while in older infants and children, theophylline plasma clearance generally exceeds adult values.[70] Studies using [^{13}C] caffeine 3-demethylation (i.e., caffeine breath test) indicate that caffeine 3-

demethylation in adolescent females declines to adult levels at Tanner stage II relative to males where it occurs at stages IV/V.[71]

In contrast to information pertaining to Phase I enzymes, the ontogeny of conjugation reactions (i.e., Phase II enzymes) is less well established. Nevertheless, available data indicate that individual glucuronosyl transferase (UGT) isoforms have unique maturational profiles. For example, bilirubin conjugation (attributed to UGT1A1) is essentially undetectable in fetal liver but increases immediately after birth reaching adult levels by 3-6 months of age. Glucuronidation of acetaminophen (a substrate for UGT1A6 and to a lesser extent, UGT1A9) is impaired in newborns and young children relative to adolescents and adults but is compensated for to some extent by increased sulfation.[72] Glucuronidation of morphine (a UGT2B7 substrate) can be detected *in vivo* in premature infants as young as 24 weeks gestational age.[73] Morphine clearance is closely correlated with post-conceptional age (PCA) and increases approximately four-fold between 27 and 40 weeks PCA necessitating dosage increases of a similar magnitude to maintain effective analgesia.[74]

A consistent observation across pediatric clinical studies is an age-dependent increase in plasma clearance for many hepatically metabolized drugs in children <10 years of age relative to adults; a finding that is often attributed as the reason for higher weight-based (mg/kg) dose requirements for young children. The mechanism(s) underlying these observed increases in plasma drug clearance are largely unknown. A single, small *in vitro* study failed to demonstrate developmental differences in hepatic CYP expression.[75] However, a detailed *in vivo* study of the CYP2C9 substrate, *S*-warfarin[76] confirmed that unbound oral clearance normalized to body weight was increased in prepubertal children but not when the data were corrected for body surface area or estimated liver weight (Figure 3). The ratio of liver to total body mass appears to reach a maximum between 1 and 3 years of age, declining to adult values during adolescence. However, antipyrine clearance which is dependent upon several CYPs[77,78] correlates significantly with age, even after correction for liver weight.[79] Therefore, it is unlikely that increased drug clearance in infants and young children can be attributed solely to a disproportionate increase in liver mass, particularly for compounds metabolized by enzymes with substantial extrahepatic expression (e.g., CYP3A4/5; UGTs).

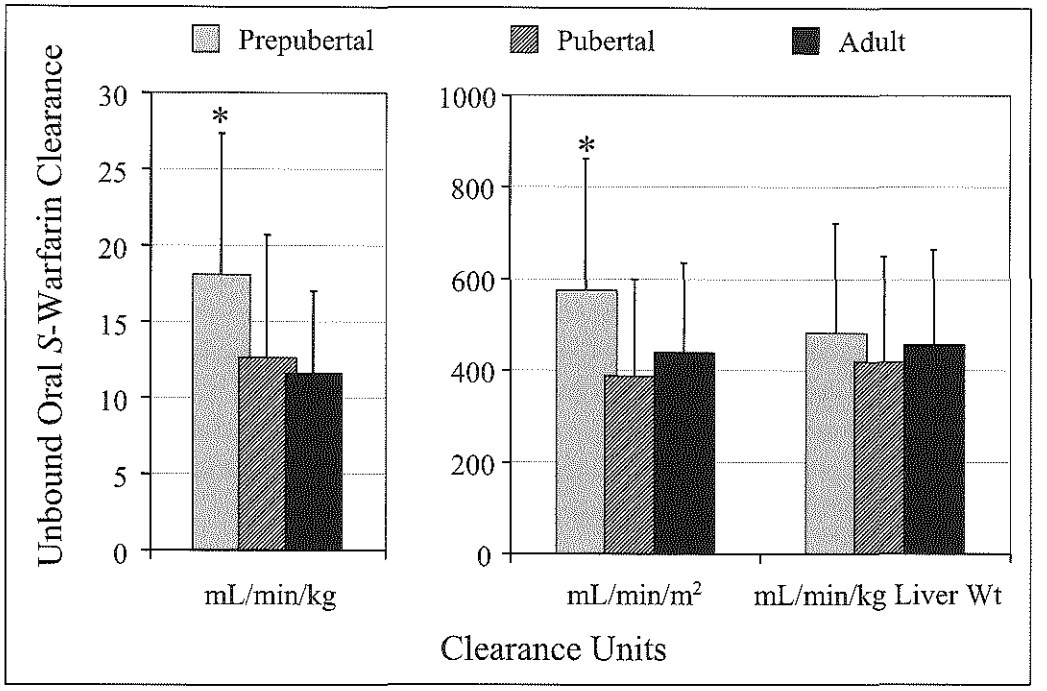

Figure 3. *Age-dependence of unbound oral S-warfarin clearance when normalized for total body weight (mL/min/kg), body surface area (ml/min/m^2) or estimated liver weight (ml/min/kg liver weight). * denotes P < 0.05 between prepubertal and the other groups (adapted from data in reference 71).*

Renal Elimination

Maturation of renal function, a dynamic process that begins early in organogenesis reaching adult capacity by early childhood, can exert a significant effect on the disposition of drugs that are primarily eliminated by the kidney. An increase in glomerular filtration rate (GFR) that occurs with maturation involves active nephrogenesis, a process that begins 9 weeks after fertilization and is complete by 36 weeks of gestation, followed by postnatal changes in renal and intrarenal blood flow.[80] GFR is low during fetal life and rises with increasing gestational age but remains constant when corrected for fetal body weight. Following birth, the GFR is 2-4 ml/min/1.73 m^2 in term neonates and as low as 0.6-0.8 ml/min/1.73 m^2 in preterm neonates. GFR increases rapidly during the first two weeks of life followed by a steady rise until adult values are reached by 8 – 12 months.[81,82] Similarly, tubular secretory pathways are immature at birth

and gain adult capacity during the first year of life. Collectively, these changes can dramatically impact renal drug clearance for compounds with extensive renal elimination. Pharmacokinetic studies of drugs primarily excreted by glomerular filtration such as ceftazidime[82] and famotidine[83] have demonstrated significant correlations between drug clearance and normal, expected maturational changes in renal function that in many instances, profoundly impact age-specific dose requirements. For example, tobramycin is eliminated almost completely by glomerular filtration resulting in dosing intervals of 36 to 48 hours in preterm infants and 24 hours in term newborns.[84] Failure to account for developmental immaturity of renal function can result in exposure to potentially toxic serum concentrations.[85] Further, concomitant medications (e.g., betamethasone, indomethacin) may alter the normal pattern of renal maturation in the neonate and as a consequence, drug disposition.[86] Thus, for drugs with extensive renal elimination, both maturational and treatment associated changes in kidney function must be considered and used to individualize treatment regimens in an age-appropriate fashion.

Pharmacodynamics

In contrast to information on pharmacokinetics, there is relatively little information available describing the impact of ontogeny on pharmacodynamics. Nonetheless, it is generally accepted that development impacts drug action and response as illustrated by a markedly increased risk of serious hepatotoxicity associated with valproic acid therapy in young infants.[87] In the case of famotidine in neonates,[83] apparent developmental differences in pharmacodynamics are directly associated with altered pharmacokinetics (i.e., age-associated reductions in GFR). However, recent data for warfarin,[76] midazolam,[88, 89] and cyclosporine[90] appear to support true age-dependent differences in the pharmacologic "targets" for drug action (e.g., warfarin, cyclosporine) and in the plasma concentration vs. effect relationship (e.g., midazolam). As is the case for drug metabolism, there may be pharmacogenetic determinants of drug action that contribute to age-dependent differences in response to drug treatment of diseases common in children (e.g., asthma, childhood leukemia).[91,92] Clearly, any assessment of pharmacodynamics in pediatric patients must consider that ontogeny can influence drug efficacy or safety by age-dependent differences in the processes that govern drug disposition (ie., determinants of exposure) and/or the milieu of events associated with drug-receptor interaction.

2. 3 The Future: Pediatric Clinical Pharmacology Research

Multiple unanswered questions regarding the influence of ontogeny on drug disposition and response offer a fertile landscape for future research. The past decade has brought about a renaissance in pediatric clinical pharmacology leading to rapid growth in research and new opportunities for therapeutic advances. Major factors included policy and funding initiatives by the NIH,[93] regulatory changes by the FDA,[94] legislation to provide incentives for inclusion of children in clinical trials,[95,96] and technological advances that have provided new research tools. Although growing interest and recent advances in pediatric pharmacology promise unprecedented opportunities, they also present considerable challenges surrounding the involvement of children in research and the lack of an established infrastructure for conducting pediatric clinical research.

While a broad based infrastructure for adult clinical pharmacology evolved during the past three decades, new drug development for children was largely ignored. Consequently, the pharmaceutical industry, federal agencies (FDA, NIH) and academic sectors found themselves with insufficient critical mass and resources to adequately embrace the new mandates for expanded pediatric pharmacology research. This situation required a rapid response from all sectors involved with pediatric drug development (i.e., industry, academia, FDA) that has produced a quantity of pediatric studies unparalleled in the history of the FDA. This is exemplified by statistics that have emanated from the pediatric provisions contained in the Food and Drug Administration Modernization Act.[94] For example, as of March 2002, the FDA had issued 241 written requests (in response to over 300 requests from pharmaceutical companies) for pediatric studies involving more than 56 drugs and well over 32,000 infants, children and adolescents. As a result, approved product labeling of 31 drugs was revised to include pediatric information for compounds used to treat a variety of conditions encountered in pediatric patients which included: cardiac arrhythmias, diabetes, gastroesophageal reflux, hypertension, HIV infection, juvenile rheumatoid arthritis, obsessive compulsive disorder, pain and seizures.[97] Label changes for several drugs (Table) made available to prescribers for the first time, critical new information that has potential significant implications for safe and effective pediatric drug use.

Table Significant Changes in Approved Product Labeling for Pediatric Drug Use

Drug	Summary of Significant Labeling Change
Gabapentin	Demonstrated need for higher doses in children < 5 years of age and new adverse events (hostility and aggression) identified in children < 12 years of age.
Propofol	Higher death rates when used for sedation in pediatric intensive care patients (9 vs. 4%) and serious bradycardia when co-administered with fentanyl
Sevoflurane	Rare incidence of seizures in pediatric patients.
Ribavirin/Intron A	Increased incidence of suicidal ideation in pediatric vs. adult patients (2.4 vs. 1%) and decreased rate of linear growth and weight gain during treatment of hepatitis C.
Pimecrolimus	Not recommended in patients < 2 years of age due to increased incidence of fever, infections and diarrhea.
Midazolam	Higher risk of serious life-threatening situations in children with congenital heart disease and pulmonary hypertension. Also, need to begin therapy with lower doses to prevent respiratory compromise in children.
Etodolac	Requirement of markedly increased (approximately 2-fold) weight-based dose to treat juvenile rheumatoid arthritis in children 6 to 16 years of age as compared to adults.
Fluvoxamine	Requirement for higher doses to treat obsessive compulsive disorder in adolescents (vs. adults); however, girls from 8 to 11 years of age may require lower doses.
Buspirone	Lack of safey and efficacy in treatment of general anxiety disorder in patients 6 to 17 years of age at recommended adult doses.
Betamethasone/ Clotrimazole	Not recommended for treatment of tinea infection in children < 17 years of age because of suppression of adrenal axis.

Adapted from data in reference 97

The expanded opportunities for pediatric drug development bring with them new challenges that will entail a need for a paradigm shift on behalf of the pharmaceutical industry, federal regulators, the NIH, and the pediatric academic community.[98] Those

conducting pediatric research must be knowledgeable about and sensitive to the special ethical issues pertaining to the participation of children in clinical trials.[98] Clinical study protocols must be designed in an age-appropriate manner to avoid unrealistic, unsafe, or unnecessary study requirements that could compromise ethical constraints and invasive study procedures that are not commonly used in the treatment of pediatric patients. Recruitment, enrollment, and retention techniques for clinical trials need to be appropriate for the age groups under study. Also, formulations of drugs must be suitable for infants and young children to insure compliance and accuracy in drug administration. Finally, subjects must be cared for in a "child friendly" environment including facilities designed for children and by health care and study personnel expert in pediatric medicine as well as clinical research.

2. 4 Conclusions

The advances in pediatric clinical pharmacology during the past decade reside with an enhanced understanding of the impact of growth and development on drug disposition and action. Compendia now exist with recommendations for drug dosing regimens tailored for age that are based upon objective, experimental evidence (e.g., pharmacokinetic and pharmacodynamic data) as opposed to anecdote and clinical convention.[99] Future progress will necessarily include research into the impact of ontogeny and pharmacogenomics on transporters, receptor systems, and cellular message systems so that the interaction of development and disease treatment can be better understood.[100] It will also require an increased commitment and investment, as well as new partnerships between pediatric academic institutions, federal agencies, and the private sector to provide the facilities and critical mass of experts sufficient to respond to the growing mandate for pediatric clinical research. As this effort moves forward, it is essential that the ultimate goal be kept clearly in sight. Specifically, providing children in our society with safe and effective drug therapy made possible by an evidence- and age-based approach to the evaluation and development of drug products essential to insure the health of pediatric patients.

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Chapter 3

Pharmacokinetics of Metoclopramide in Neonates

Based on the article:
Pharmacokinetics of Metoclopramide in Neonates
Kearns GL, van den Anker JN, Reed MD, Blumer JL.
J Clin Pharmacol 37:122-128 (1998)

3.1 Abstract

Despite of its wide use as a prokinetic agent in neonates and infants with gastroesophageal reflux (GER), the pharmacokinetics of metoclopramide have not been characterized in this pediatric subpopulation. A single-dose pharmacokinetic study of oral metoclopramide (0.1 to 0.15 mg/kg) was performed in 10 fasted premature infants (weight 1.1 to 3.2 kg) ranging from 31 to 40 weeks postconceptional age. Metoclopramide was quantitated from repeated blood samples ($n = 9$ over 24 hours) by high-performance liquid chromatografy. A one-compartment open model with first-order absorption best described the plasma concentration-time data. No correlations were observed between gestational, postnatal or postconceptional age and any of the pharmacokinetic parameters studied. Comparison of the pharmacokinetic parameters from the study cohort and those reported previously from a similar study of older infants revealed no statistically significant differences. However, a prolonged apparent plasma clearance (Cl/F) of metoclopramide was observed in 30% of the infants studied, and the mean Cl/F and apparent steady-state volume of distribution (Vd_{ss}/F) were approximately 1.4- and 2.1-fold higher, respectively, than values reported in previous studies of metoclopramide disposition in adults. These data suggest that metoclopramide pharmacokinetics may exhibit a developmental dependency. Thus, a metoclopramide dose of 0.15 mg/kg given orally, every 6 hours is recommended for the initiation of prokinetic therapy with this agent in infants who are ≥ 31 weeks postconceptional age.

3.2 Introduction

Gastroesophageal reflux (GER) is common in infants and newborns and is usually treated with thickened feedings, positional therapy and prokinetic drugs.[1,2] Despite the wide spread use of prokinetic agents in both neonates and infants, none of these agents are labeled for the treatment of GER within this patient subpopulation. Nonetheless, off-label use of prokinetic agents has become common place in neonates and young infants. In many instances, a "patient specific" therapeutic trial of these agents is undertaken in infants with a presumptive diagnosis of GER disease, often made on the basis of clinical evidence of regurgitation associated with feeding. Recently, Pettignano et al.[3] highlighted the proarrhythmic effects of cisapride in infants and children receiving concomitant

therapy with erythromycin, a CYP3A4 inhibitor.[4] Clinically important adverse events such as these associated with cisapride has led to a resurgence of interest in the use of metoclopramide for treating of GER.

Metoclopramide was developed in France as a derivative of orthoprocainamide in the early 1960s and has been used in the United States for the treatment of GER in infants since the 1980s. Metoclopramide was the first of the benzamides with effects on the central nervous system and the gastrointestinal tract (e.g., increasing lower esophageal sphincter pressure and improving gastroduodenal coordination), both of which are believed to be caused by modulation of acetylcholine and serotonin.[5,6] The pharmacokinetics and pharmacodynamics of metoclopramide have been examined in children[7] and infants.[8] However, only the pharmacodynamics of this drug have been evaluated to date in preterm infants.[9]

In view of the extensive hepatic metabolism of metoclopramide both via N-4-sulfate and N-glucuronide conjugation,[5] and the ontogeny of phase II enzymes in humans,[4] developmental changes in the disposition of metoclopramide would be anticipated. Given the fact that as many as 50% of patients receiving metoclopramide may experience CNS-related adverse effects,[6,7] it is reasonable to consider the potential impact of factors such as development on the disposition of metoclopramide and in turn, the dose vs. concentration vs. effect relationship. We therefore studied the pharmacokinetics of metoclopramide in preterm infants.

3.3 Patients and Methods

Patients

Ten preterm infants (9 boys, 1 girl, 31-40 weeks postconceptional age) with a diagnosis of GER made on clinical grounds were entered into this study. All infants had normal values for hemoglobin and hematocrit, normal hepatic and renal function, and none had experienced an episode of clinically significant hypoperfusion requiring fluid and/or pharmacologic resuscitation. The decision to institute metoclopramide therapy was made independent of the study protocol by an attending neonatologist. Informed parental consent was obtained before patient enrollment. The experimental protocol was approved

by the Human Research Advisory Committee of the University of Arkansas for Medical Sciences, and subjects were enrolled by informed parental consent.

Drug Administration and Sampling

A single oral dose of metoclopramide (0.1 or 0.15 mg/kg) was given as a solution formulation after a 1.5-hour period of fasting via oral syringe or nasogastric tube, followed by 10 ml of distilled water (25°C). Age-appropriate feedings (i.e., age and weight appropriate volumes of either infant formula or breast milk) were reinstituted 1.5 hours following the metoclopramide dose.

Repeated blood samples (0.3 ml each) for quantitation of metoclopramide were drawn from indwelling umbilical vein catheters placed before metoclopramide dosing for non-study related medical care. Sample collection was performed immediately before and at 0.5, 1, 2, 3, 6, 12, 18, and 24 hours after the dose. Blood samples were allowed to clot in polyethylene tubes at 25°C for 45 minutes. Serum was separated following centrifugation at 5,000 X g for five minutes and was stored at -70°C until analysis.

Analytical Methods

Quantitation of metoclopramide from serum was performed using a reverse-phase high-pressure-liquid chromatography (HPLC) method[10] on an automated HPLC system (Waters Corporation, Millford, MA). Briefly, serum samples (150 µl) were combined with 50 µl of 5 N NaOH, 25 µl of internal standard (quinidine), and 3.0 ml of methyl t-butyl ether. After extraction, the organic phase was evaporated under N_2 and was reconstituted with mobile phase (acetate/methanol buffer). Chromatography was carried out using a Resolve CN precolumn (Waters Corporation) and a Phenomenex IB SIL 5CN (240 x 4.6 mm) analytical column (Phenomenex, Torrence, CA). The eluate was monitored at 309 nm and 0.001 AUFS where the retention times for metoclopramide and quinidine were 12.5 and 17 minutes, respectively. The range of linearity for the method was 6.25 to 200 ng/ml, the limit of detection was 3.13 ng/ml, and the coefficients of variation for intra- and interday reproducibility were consistently < 10% at 10, 50 and 100 ng/ml. All reagents used for the HPLC assay were purchased commercially (Sigma Chemical Company, St. Louis, MO) and were of the highest grade obtainable.

Data Analysis

Individual metoclopramide serum concentration data were curve fit using a peeling algorithm,[11] which yielded initial polyexponential parameter estimates. Final parameters were generated using a weighted least-squares algorithm[12] with weight set as the reciprocal of the calculated plasma concentration. Compartmental model selection was made using Akaike's Information Criterion,[13] and the coefficients of variation for parameters estimated in the model. The apparent first-order elimination rate constant was then determined from the best fit of a given data set. The area under the plasma concentration-time curve (AUC) was generated using the linear trapezoidal rule which, was extrapolated to infinity using the final plasma concentration calculated from the curve fit. The apparent total plasma clearance (Cl/F) and apparent steady-state volume of distribution (Vd_{ss}/F) were calculated using a noncompartmental approach, as previously described.[14] Relationships between apparent plasma clearance (Cl/F) and steady state volume of distribution (Vd_{ss}/F), and postnatal and postconceptional age were evaluated using nonlinear regression analysis. Comparison of pharmacokinetic parameters between the present and previous study was undertaken using a two-tailed, unpaired Student *t* test. All data analyses were accomplished using software packages (Siphar/Base, version 4.0 and S-Stat) available from SIMED (Creitel-Cedex, France). The level of significance accepted for all analyses was $\alpha = 0.05$.

Additionally, comparison of data for both Cl/F and Vd_{ss}/F between this present study and a previous study of metoclopramide pharmacokinetics in infants[8] was performed. For reference and comparison purposes, pharmacokinetic parameters from selected pharmacokinetic studies of intravenous metoclopramide in adult subjects[5] were also examined in comparison to the experimental data from our study population. Throughout the manuscript, descriptive demographic and pharmacokinetic data are presented as the mean ± 1 standard deviation of the mean and the range unless otherwise denoted.

3.4 Results

Nine boys and one girl ranging from 31 to 40 weeks postconceptional age (35.3 ± 2.7 weeks) were studied. The subjects ranged in gestational age from 26 to 36 weeks (31.2 ±

3.2 weeks), in postnatal age from 1 to 7 weeks (4.1 ± 1.9 weeks) and in weight from 1.14 to 3.20 kg (1.87 ± 0.57 kg). Five infants were black and five were white. All infants were prescribed metoclopramide before entry into the study for symptomatic treatment of excessive regurgitation associated with feeding. The demographic data for the studied infants are presented in Table 1.

Table 1 Demographic Characteristics

Patient No	Race	Gender	weight (kg)	Gestational Age (wks)	Postnatal Age (wks)	Postconceptional Age (wks)
1	W	M	1.97	31	7	38
2	B	M	1.75	31	4	35
3	W	M	1.69	34	2	36
4	W	F	1.36	26	7	33
5	B	M	3.20	36	4	40
6	W	M	1.70	30	3	33
7	W	M	1.14	27	4	31
8	B	M	2.27	35	1	36
9	B	M	1.58	30	4	34
10	B	M	2.07	32	5	37
Mean			1.87	31.2	4.1	35.3
SD			0.57	3.22	1.9	2.67

Metoclopramide could be quantitated in postdose serum samples in all subjects through 3 hours. Additionally, the drug was measurable in 9 of 10 infants at 6 hours, in 6 of 10 at 12 hours, in 4 of 10 at 18 hours and in 2 of 10 at 24 hours following drug administration. Of the two subjects with quantifiable concentrations at 24 hours, one (patient 5) had a metoclopramide concentration of 15.5 ng/ml at 24 hours and the other (patient 6) a concentration of 2.5 ng/ml. The mean (± SEM) serum concentration- time data for the study population are illustrated in the Figure 1. The apparent peak concentration of metoclopramide in plasma (C_{max} = 17.7 ± 6.2, range 8.9 - 29.2 ng/ml) and the apparent time of peak serum concentration (T_{max} = 2.5 ± 0.7, range 0.5 - 4 hrs) were not found to correlate with gestational age, postnatal age or postconceptional age.

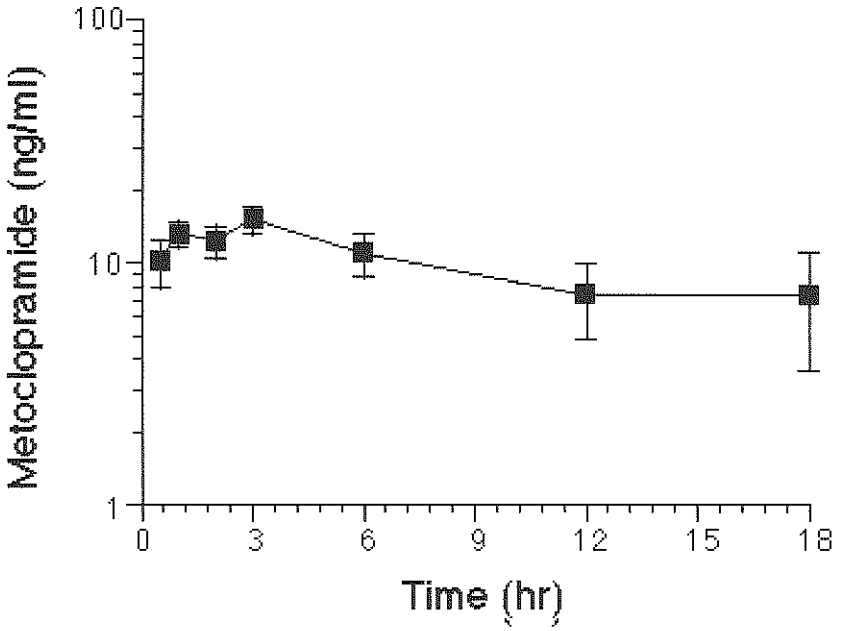

Figure 1 *Mean ± SEM metoclopramide plasma concentration-time data in nine preterm infants after administration of a single oral dose of metoclopramide solution.*

Curve fitting of both individual and mean serum concentration-time data revealed that a one-compartment open model with first-order absorption best described metoclopramide disposition over the 24- hour postdose sampling period. One neonate (patient 9) had metoclopramide serum concentrations below the limit of quantification in the 12-through 24-hour postdose sampling interval; thus accurate estimation of the apparent terminal elimination rate constant and other pharmacokinetic parameters was not possible.

The pharmacokinetic parameters for each infant are presented and summarized in Table 2. To examine the potential influence of development on metoclopramide disposition in our study cohort, correlations between age (i.e., gestational age, postnatal age, and postconceptional age) and the elimination rate constant, Cl/F and $Vd_{SS}/F_{ss}/F$ were performed. All of these evaluations failed to reveal any statistically significant correlation (linear or non-linear).

Table 2 Metoclopramide Pharmacokinetic Parameters in Neonates*

Patient No.	Dose (µg/kg)	Ke (1/hr)	Ka (1/hr)	AUC_{corr} (ng/ml.hr)	C_{max} (ng/ml)	T_{max} (hrs)	Cl/F (l/hr/kg)	Vd_s/F (l/kg)
1	100	0.157	0.489	258.83	16	3	0.386	5.31
2	100	0.247	1.72	100.29	20	1	0.997	4.70
3	100	0.062	1.40	225.54	12	2	0.443	7.53
4	100	0.065	0.63	321.56	16	3	0.310	5.11
5	150	0.030	1.08	647.63	29.2	4	0.154	5.14
6	100	0.074	1.61	163.15	13.7	2	0.612	9.38
7	100	0.222	ND	101.33	25.9	0.5	0.986	4.95
8	150	0.221	0.257	41.130	8.9	3	2.43	10.54
9	150	ND	ND	ND	ND	ND	ND	ND
10	150	0.082	7.70	119.37	15.4	3	0.84	9.78
Mean	120	0.129	1.86	219.87	17.72	2.45	0.795	6.94
SD	25.82	0.083	2.42	183.20	6.20	1.07	0.682	2.39

** The elimination and absorption half-life for metoclopramide determined from the mean values of Ke and Ka were 5.4 and 0.37 hours, respectively.*

Ke, apparent elimination rate constant; Ka, apparent absorption rate constant; AUC_{corr}, area under the serum concentration-time curve corrected for a metoclopramide dose of 100 µg/kg; C_{max}, apparent peak serum concentration; T_{max}, time of Cmax; Cl/F, apparent total serum clearance uncorrected for extent of absorption; Vd_{SS}/F, apparent steady-state volume of distribution uncorrected for absorption; ND, not determined due to insufficient points on the absorption phase (patient 7) or insufficient serum concentration observations to accurately characterize the elimination phase (patient 9).

Table 3 Age-Specific Comparison of Metoclopramide Pharmacokinetics

Study (reference)	Population	Route of Administration	Cl/F (L/h/kg)	Vd_{SS}(l/kg)
Kearns et al 1998[4]	Neonates	oral	0.79 ± 0.68	6.94 ± 2.39
Kearns et al 1988[8]	infants	oral	0.66 ± 0.39	4.94 ± 1.06
Ross-Lee et al 1981[15]*	adults	intravenous	0.53 ± 0.19	3.43 ± 1.18
Block et al 1980[16]*	adults	intravenous	0.55 ± 0.12	3.10 ± 0.64

** Data from Ross-Lee et al[15] reflects a 10 mg metoclopramide dose, while that from Block et al[16] reflects a 20 mg dose.*

Data are presented as mean ± standard deviation. Comparison of both Cl/F and Vd_{SS}/F between neonatal and infant studies revealed $P > 0.05$ ($P = 0.25$ for Cl/F and 0.07 for Vd_{ss}/F) Cl/F, apparent total serum clearance uncorrected for extent of absorption; Vd_{ss}/F apparent steady-state volume of distribution uncorrected for absorption.

Comparison of data for both Cl/F and Vd_{ss}/F between this study and a previous study of metoclopramide pharmacokinetics in infants[8] together with data from previously published pharmacokinetic studies of intravenous metoclopramide[15,16] are summarized in Table 3. In this comparison, the mean values for metoclopramide Cl/F and Vd_{ss}/F in preterm infants were not significantly different from those reported in a previous study[8] of six infants aged 1.0 to 5.5 months. However, they were approximately 1.4 (for Cl/F) and 2.1 (for Vd_{ss}/F) fold higher than reported from previous studies in adults.[15,16]

3.5 Discussion

Gastroesophageal reflux (GER) is a common disorder in infants less than 6 months of age.[1,9] Symptomatic improvement of this disorder following treatment with metoclopramide has been reported with intravenous doses ranging from 0.1 mg/kg/day in neonates[9] to 0.3 mg/kg intramuscularly per dose in infants[17] Several subsequent studies have reported beneficial clinical and pharmacodynamic effects of oral metoclopramide in infants with GER without gastroparesis and feeding intolerance.[18-22] In contrast, other investigations have failed to demonstrate the efficacy of metoclopramide in the treatment of GER reflux in infants with oral doses ranging from 0.5 to 0.6 mg/kg/day.[23,24]

Hyams et al.[25] have suggested that escalation of the metoclopramide dose to 0.3 mg/kg per dose (i.e., 0.9 to 1.2 mg/kg/day) may be required for demonstrable effects on esophageal acid clearance in infants with GER; thereby implying a concentration-effect relationship for metoclopramide. It should be noted, however, that the dose of metoclopramide for GER in newborn infants has not been determined from studies examining the combined pharmacokinetics and pharmacodynamics of this agent in this particular pediatric subpopulation, but rather has been inferred from limited pharmacokinetic data in older infants and children[5,8] and objective measurements (e.g., esophageal pH monitoring, esophageal acid clearance, frequency/volume of regurgitated feedings) from previous clinical trials.[9,17-25]

Kearns et al.[8] reported results from an investigation of the pharmacokinetics and pharmacodynamics of metoclopramide in six infants (1.0 to 5.5 months) with GER confirmed by esophageal pH monitoring. This study showed that after an oral dose of

0.15 mg/kg given every 6 hours, the mean elimination serum half-life for metoclopramide after the tenth dose was 4.2 hours. In addition, beneficial effects (as determined by continuous esophageal pH monitoring) were associated with steady-state C_{max} and predose (i.e., trough) plasma concentration values of 56 ± 23.5 and 32.7 ± 13.2 ng/ml, respectively. These findings were corroborated by a study of 24 infants, aged 1 to 18 months with GER, which demonstrated significant reductions in GER in association with metoclopramide plasma concentrations between 20 and 90 ng/ml.[26]

The apparent adequacy of a simple one-compartment model to characterize the pharmacokinetics of metoclopramide in our study population is in agreement with previous data demonstrating linear pharmacokinetics of metoclopramide over a threefold range of doses.[5,8] As illustrated by our data (Figure 1, Table 2), metoclopramide absorption was moderately rapid with attainment of apparent peak plasma concentrations approximately 0.5 to 4 hours following a dose.

As expected in a population of preterm neonates, the variability in the pharmacokinetic parameters (Table 2) was greater than that observed previously in older infants with GER (Table 3). While a comparison of the mean Cl/F and Vd_{ss}/F for metoclopramide between our population of preterm infants and an older cohort of infants studied previously[8] failed to demonstrate statistically significant differences for either of these pharmacokinetic parameters, the values (for Cl/F and Vd_{ss}/F) in the preterm infants (Table 3) were significantly greater than values reported for these parameters from previously reported studies in adults.[15,16] Despite the absence of a correlation between metoclopramide pharmacokinetic parameters and age in our study cohort, it is important to note that 3 of the 10 infants studied had an elimination half-life of more than 10 hours (range 10.5 to 23.1 hours).

To illustrate the potential importance of an increased elimination half-life for metoclopramide in the neonate, we used a simple one-compartment open model with first-order absorption and average pharmacokinetic parameters from our entire study population (e.g., Ka, Ke and Vd_{ss}/F) (Table 2) as well as a fixed estimate of elimination half-life (i.e., 11 hours) to simulate steady-state peak (C_{max}) and trough (C_{min}) metoclopramide plasma concentrations for two common oral dosing regimens. As illustrated by the simulated plasma concentrations (Table 4), the consequence of

administering "standard" metoclopramide doses (i.e., 0.2 mg/kg every 6hours) to an infant with a prolonged elimination half-life are almost two- and three-fold increases in C_{max} and C_{min}, respectively, and an approximate two-fold increase in the AUC for a 6-hour dosing interval. Although not specifically evaluable from the data collected in this study or from previous pediatric investigations of metoclopramide reported in the literature, it appears reasonable to speculate that in those preterm neonates with prolonged plasma clearance, "standard" doses of the drug could easily result in the accumulation of steady-state serum concentrations, which possibly could produce toxicity.

Although the seemingly increased apparent volume of distribution for metoclopramide in our preterm infants might be explained in part by the physicochemical characteristics of the drug[5] and known developmental changes in body composition,[27] the same is not true for the findings relative to plasma clearance (Table 3). It is possible that "enhanced" plasma clearance of metoclopramide in some neonates and in infants may represent developmental variations in isoform-specific sulfotransferase activity responsible for the N-4-sulfation of metoclopramide; a pathway responsible for a major portion (32%) of the drug's metabolism in humans.[5] This phenomenon (enhanced sulfation) has been previously described for acetaminophen metabolism in infants[28] and demonstrates the potential importance of developmental differences in the ontogeny of phase II enzymes.

In contrast, the apparent delayed plasma clearance in 30% of our preterm infants, which did not appear to be associated with age (Table 2) may not represent deficient sulfotransferase activity associated with immaturity but rather, normal pharmacogenetic variability (e.g., a poor-metabolizer phenotype) for sulfotransferase isoforms as has recently been reported by Weinshilboum et al.[29] As illustrated by our pharmacokinetic simulations (Table 4), the administration of repeated doses of metoclopramide previously recommended for the management of GER in these neonates could easily result in metoclopramide plasma concentrations capable of increasing the risk of adverse drug effects. Thus, the pharmacokinetic data from our study cohort (Table 2) and the aforementioned simulations (Table 4) would appear to support the initiation of metoclopramide therapy in the neonate who is >31 weeks postconceptional age with an oral dose of 0.15 mg/kg given every 6 hours.

Table 4 Simulated Steady State Metoclopramide Serum Concentrations in Pre-term Infants

Dose Regimen	C_{max} (ng/ml)	C_{min} (ng/ml)	AUC_{ss} (ng/ml.hr)
0.15 mg/kg every 6 hours	32.3	19.3	154.8
0.20 mg/kg every 6 hours	43.4	26.1	208.5
0.20 mg/kg every 6 hours*	78.1		63.1

**Denotes simulation performed for maximal recommended dose using an elimination $t_{1/2}$ of 11 hours. All other simulations used mean values for Ke, Ka, and Vd_{ss}/F for metoclopramide from the study cohort (Table 2)*
C_{max}, peak concentration; C_{min}, trough concentration; AUC_{ss}, steady state area under the serum concentration-time curve for a dosing interval.

Metoclopramide use in the management of GER and feeding intolerance in neonates remains a common clinical practice in the United states and Europe. The data from this investigation demonstrate the potential for developmental dependence, and perhaps normal heterogeneity in the biotransformation of metoclopramide, to have a sufficient impact on the pharmacokinetics of this drug so as to alter the dose-concentration-effect relationship. Until future studies are available to more clearly define the pharmacokinetic-pharmacodynamic interface for metoclopramide in both preterm and term neonates, attention to the potential impact of ontogeny on the drug's disposition should be considered in the decision to prescribe therapy in this pediatric subpopulation and in monitoring these patients for metoclopramide-associated adverse effects.

3.6 Acknowledgements

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Chapter 4

Single Dose Pharmacokinetics of Linezolid in Infants and Children

based on the article:

Single Dose Pharmacokinetics of Linezolid in Infants and Children

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4.1 Abstract

Background
Linezolid is an oxazolidinone antibiotic with excellent *in vitro* activity against a number of Gram-positive organisms including antibiotic-resistant isolates. The safety and pharmacokinetics of intravenously administered linezolid were evaluated in children and adolescents to examine the potential for developmental dependence on its disposition characteristics.

Methods
Fifty-eight children (3 months to16 years old) participated in this study; 44 received a single 1.5-mg/kg dose and 14 received a single 10-mg/kg dose of linezolid administered by intravenous infusion. Repeated blood samples ($n = 10$ in children $\geq$ 12 months; $n = 8$ in children 3-12 months) were obtained over 24 h after drug administration, and linezolid was quantitated from plasma by high performance liquid chromatography with mass spectrometry detection. Plasma concentration *vs.* time data were evaluated with a model independent approach.

Results
Linezolid was well-tolerated by all subjects. The disposition of linezolid appears to be age-dependent. A significant although weak correlation between age and total body clearance was observed. The mean (±SD) values for elimination half-life, total clearance and apparent volume of distribution were 3.0 ± 1.1 h, 0.34 ± 0.15 liter/h/kg and 0.73 ± 0.18 liter/kg, respectively. Estimates of total body clearance and volume of distribution were significantly greater in children than historical values from adult data. As such maximum achievable linezolid plasma concentrations were slightly lower in children, and concentrations 12 h after a single 10-mg/kg dose were below the MIC_{90} for selected pathogens with *in vitro* sensitivity to the drug.

Conclusion
Based on these data, a linezolid dose of 10 mg/kg given two to three times daily would appear appropriate for use in pediatric therapeutic clinical trials of this agent.

4.2 Introduction

The rise of antibiotic resistance among Gram-positive bacteria has emerged as a significant clinical concern worldwide, with prevalence estimates of betalactam and glycopeptide resistance demonstrating a marked increase over the past decade.[1] Between 1990 and 1997, vancomycin-resistant isolates of *Enterococcus* spp. increased from <1% to 18% and penicillin-resistant isolates of *S. pneumoniae* from 4% to 31-42%.[2] Resistant organisms are now commonly isolated from patients with infections across a wide spectrum of ages. When it occurs, multiple drug resistance can result in few to no available therapeutic alternatives from among currently marketed antimicrobial agents.

Linezolid, [(S)-N-{{3-[3-fluoro-4-(4-morpholinyl)phenyl)]}-2-oxo-5-oxazolidinyl}methyl}-acetamide],is a new antibiotic of the oxazolidinone class that demonstrates considerable potential in the treatment of multiply resistant Gram-positive organisms. The oxazolidinones are a totally synthetic class of antibiotics first patented in 1978 and later described in 1987 as potential candidates for the treatment of human infections.[3]

Linezolid is the newest member of this class of compounds that appears to have overcome limitations in toxicity associated with earlier oxazolindones. Currently the drug is marketed in the US and is undergoing continued clinical trials in both adult and pediatric patients. Linezolid binds to the 50S ribosomal subunit and inhibits bacterial growth via inhibition of protein synthesis. Although these agents share a similar binding site with chloramphenicol and lincomycin, their mechanisms of action appear to be unique.[4] Linezolid does not inhibit the formation of initiator transfer-RNA or peptidyltransferase and has no effect on the termination reaction.[4,5] Although the specific mechanism of action for linezolid remains to be completely characterized, it appears to occur early in the process of protein synthesis, possibly distorting the binding site for the initiation transfer RNA.[6] *In vitro*, linezolid possesses excellent activity against a number of Gram-positive organisms, regardless of the betalactam or glycopeptide resistance phenotype. The range of concentrations required to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of select clinical isolates are provided in Table 1.[1, 7-14]

Table 1. Range of reported minimum inhibitory concentrations for linezolid against select clinical isolates.[1,7-14]

Organism	*n*	MIC_{50} (mg/l)	MIC_{90} (mg/l)	Range
Staphylococcus aureus				
Penicillin-susceptible	17	2	2	1-2
Methicillin-susceptible	223	1-2	2-4	0.5-8
Methicillin-susceptible/ciprofloxacin-resistant	16	1	2	0.5-2
Methicillin-resistant	339	1-2	2-4	0.5-16
Methicillin-resistant/ciprofloxacin-resistant	189	1	2	0.12-2
Staphylococcus epidermidis				
Methicillin-susceptible	75	0.25-2	0.5-2	0.12-2
Methicillin-resistant	77	1	2	0.5-4
Coagulase-negative *Staphylococcus* (non epi)				
Methicillin-susceptible	25	1	2	0.5-2
Methicillin-resistant	16	1	2	1-2
Staphylococcus hemolyticus				
Methicillin-susceptible	16	1	1	0.5-1
Methicillin-resistant	43	1	1	0.5-1
Staphylococcus saprophyticus	30	1	1	1
Staphylococcus pneumoniae	50	2	2	1-2
Penicillin-sensitive	10	0.5	1	0.5-1
Penicillin-intermediate/resistant	30	1	1	<0.125-2
Staphylococcus pyogenes	30	1	1-2	0.5-2
Group B Streptococci	50	1	1	1
Streptococcus milleri	28	2	2	1-2
Enterococcus faecalis				
Vancomycin-sensitive	88	1-2	1-4	0.25-4
Vancomycin-resistant (Van B)	10	2	4	2-4
Enterococcus faecium				
Vancomycin-sensitive	84	1-2	1-2	0.25-4
Vancomycin-resistant (Van A)	16	2	2-4	1-4
Vancomycin-resistant (Van B)	14	2	4	0.5-4
Enterococcus gallinarium [Vancomycin-resistant (Van C1)]				2-4
Enterococcus casseliflavus [Vancomycin-resistant (Van C2/3)]				2-4
Bacteroides fragilis	35	4	4-8	1-8
Clostidium perfringens	9	2	2	1-2
Clostidium dificile	9	8	8	8
Corynebacterium	11	0.5	0.5	0.5-1
Listeria monocytogenes	10	2	2	2-4
Moraxella catarrhalis	10	4	4	4-8

Given ever increasing concerns for infections caused by multiply resistant organisms in infants and children and the potential for linezolid to become an important antimicrobial agent in pediatrics, we conducted a Phase I single-dose pharmacokinetic study of the intravenous formulation of linezolid in infants and children. The objective of this investigation was to examine the potential for developmental dependence in linezolid pharmacokinetics and to compare its disposition characteristics relative to those previously reported from pharmacokinetic studies in adults.

4.3 Methods

Subjects

Fifty-eight infants and children were enrolled in this open label, multicenter clinical investigation. Subjects were eligible for enrollment if they met the following inclusion criteria:

1. age between 3 months and 18 years,
2. body weight greater than the 5th and less than the 95th percentile for age and gender and
3. the availability of parents and/or legal guardians for the purpose of obtaining informed consent.

Additionally, female subjects who had attained menarche were required to have a negative serum pregnancy test prior to administration of the study drug.

Subjects were excluded from participation if any of the following criteria were met:

1. evidence of a chronic disease state as reflected by medical history, physical examination, and/or abnormal laboratory test results reflecting either hepatic or renal compromise, or the presence of anemia,
2. a history of drug or alcohol abuse within the past 12 months and/or a positive urine drug screen,
3. receipt of an investigational drug administered as part of a clinical trial within 30 days of study drug administration,
4. use of a prescription drug known to induce or inhibit cytochromes P450 and
5. inability to tolerate required study procedures (e.g., maintaining vascular access sufficient to enable repeated blood sampling).

A medical history, physical examination, and clinical laboratory tests (complete blood count, serum chemistry panel, liver function tests and urinalysis) were performed in each subject prior to enrollment and within 24 hours of study completion. All subjects were enrolled after informed parental consent and patient assent when appropriate (i.e., ≥ 7 years of age). The study protocol was approved by the Institutional Review Boards of the participating institutions; all of which were members of the Pediatric Pharmacology Research Unit (PPRU) Network.

Study Design

The study was conducted as an open label, single dose evaluation of linezolid pharmacokinetics at two doses. The safety and pharmacokinetics of intravenously administered linezolid were evaluated initially in 44 patients receiving a single 1.5-mg/kg dose followed by the evaluation of an additional 14 patients receiving a dose of 10 mg/kg (maximum 600 mg). Subjects received linezolid (Linezolid sterile solution, 2 mg/mL, Pharmacia & Upjohn Co., Kalamazoo, MI) as an intravenous, constant rate infusion delivered 30 min by infusion pump with microbore tubing. All subjects were studied during an inpatient stay at the participating institutions where they remained throughout the 24-h postdose sample collection and observation period.

Sample Collection

Blood samples for determination of linezolid concentration were collected from an indwelling venous catheter. Samples (1.5 ml each) were collected from children (≥ 12 months) receiving the 1.5 mg/kg dose immediately before drug administration and at 0.5, 0.6, 0.75, 1, 2, 4, 8, 12 and 24 h after the beginning of the infusion. Children < 12 months of age were sampled according to a less intensive scheme with exclusion of the 0.5- and 0.75-h samples. A smaller blood sample volume (0.75 ml each) was collected from children receiving the 10-mg/kg dose with sampling immediately before dosing and at 0.6, 1, 2, 4, 8, 12 and 24 h after the start of the infusion. Plasma was separated by centrifugation and stored at -70°C until analysis. In addition urine was collected from all patients capable of voiding in sterile urine collection containers and from infants in whom an indwelling catheter was in place for therapeutic indications not related to study participation. Urine sampling was conducted immediately before linezolid administration and between 0 and 4, 4 and 8, 8 and 12 and 12 and 24 h after dosing. Quantitative urine

collections were mixed, the total volume and pH determined and a 25-ml aliquot was frozen at -70°C until analysis.

Analytical Procedures

Linezolid plasma concentrations were determined using a validated high performance liquid chromatographic assay as described previously.[15] The eluant was monitored with an atmospheric pressure chemical ionization interface to a triple quadrapole mass spectrometer that was operating in a positive selective ion monitoring mode. Linezolid urine concentrations were determined with a validated high performance liquid chromatographic assay with ultraviolet detection ($\lambda_{251\ nm}$) described previously.[16]

An eight point standard curve using the ratio of active compound to internal standard was prepared daily and was used to calculate all plasma and urine linezolid concentrations. The limit of quantitation was established at 1 ng/ml for the plasma assay and 0.4 μg/ml for the urine assay. The analytical method demonstrated linearity at plasma linezolid concentrations ranging from 1 to 250 ng/ml ($r^2 > 0.998$) and urine linezolid concentrations ranging from 0.2 to 100 μg/ml ($r^2 > 0.998$). For both assays interday and intraday assay variability was consistently $\leq$ 6.0% and 5.0%, respectively for concentrations within the range of linearity. All assays were performed by an independent laboratory (AvTech Laboratories, Inc., Kalamazoo, MI). The mean linezolid concentrations from the analysis of duplicate samples was reported and used to accomplish the pharmacokinetic and statistical analyses.

Pharmacokinetic and Statistical Analysis

Pharmacokinetic and statistical analyses were conducted using the Clinical Pharmacokinetics Analysis Package Version 1.0 (Trilogy, Location) and SAS version 6.0 (SAS Institute, Inc., Cary, NC). All data were evaluated using a model-independent approach. Individual C_{max} and T_{max} were determined by visual inspection of the plasma concentration *vs.* time profile. The area under the plasma concentration *vs.* time curve from 0 to 24 h postdose ($AUC_{0\text{-}24}$) was determined using the log-linear trapezoidal rule. Extrapolation of the AUC to infinity ($AUC_{0\text{-}\infty}$) was calculated by summation of $AUC_{0\text{-}24}$ + Cp_{24}/λ_i, where Cp_{24} represented the predicted plasma concentration at 24 hours postdose and λ_i is the apparent terminal elimination rate constant. Total body clearance (Cl) and steady state apparent volume of distribution (Vd_{ss}) were calculated from the $AUC_{0\text{-}\infty}$.

Linezolid pharmacokinetic parameters for the study cohort were examined using standard descriptive statistics (i.e., mean, SD, range). Examination of the data for possible age dependence in the linezolid pharmacokinetic parameters (e.g., Cl, Vd_{ss}, dose-normalized $AUC_{0-\infty}$, dose normalized C_{max}) was undertaken using univariate analysis of variance and both linear and nonlinear least squares regression models. Comparison of linezolid pharmacokinetic parameters from the study population to those from a similar study conducted in healthy adults (data on file, Pharmacia & Upjohn Co., Inc., Kalamazoo, MI data on file) was performed using the Wilk-Shapiro test to assess homogeneity of variance, followed by a two tailed, unpaired Student *t* test. The significance limit accepted for all statistical analyses was $\alpha = 0.05$.

4.4 Results

Fifty-seven subjects completed the study protocol. Sufficient data to determine pharmacokinetic parameters were available in 54 of these: 40 in the low dose (1.5-mg/kg) group and 14 in the high dose (10-mg/kg) group. Pharmacokinetic parameters were not available for 3 children due to a delayed C_{max} occurring at 4 h in 1 child and insufficient sampling to accurately estimate $AUC_{0-\infty}$, in the remaining 2 children. Demographic data for the evaluable subjects are provided in Table 2. Linezolid was well tolerated in the pediatric subjects with only mild adverse events reported in 6 patients during the study period; 4 in the 1.5-mg/kg dose group and 2 in the 10-mg/kg dose group. These adverse events consisted of redness or burning at the catheter site ($n = 2$), rash ($n = 3$) and raised papillae on the tongue ($n = 1$), all of which required no specific treatment.

The mean (+SD) plasma linezolid concentration *vs.* time data for linezolid during the 24-h postdose period for both dosing groups are illustrated in Figure 1. As expected, based on the broad age range of subjects evaluated, the plasma concentration data reflected considerable intersubject variability for dose-normalized C_{max} (0.10 ± 0.06; range, 0.02-0.22 μg/ml). Linezolid plasma concentrations observed in the 10-mg/kg group at both 12 (0.33 ± 0.07 mg/l) and 24 h (0.07 ± 0.14 mg/l) post-dose were well below the reported MIC_{50} for most organisms of interest (Table 1).

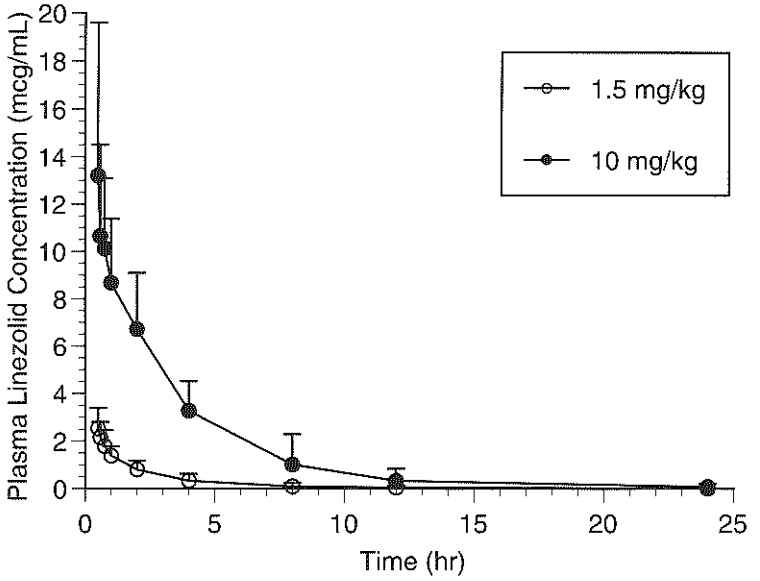

Figure 1 *Mean (+ SD) plasma concentration vs. time profile in 40 pediatric patients given a single 1.5-mg/kg dose and 14 pediatric patients given a 10-mg/kg dose of linezolid.*

The mean ± SD and range of pharmacokinetic parameters for both dose groups of linezolid are shown in Table 2. No statistically significant differences were detected between the 1.5- and 10.0-mg/kg dose groups with respect to subject demographic and pharmacokinetic parameters when normalized for dose as appropriate. A significant, non-linear correlation between age and total body clearance of linezolid ($r^2 = 0.24$, $P = 0.004$) was observed for children between 3 months and 16 years of age (Figure 2a) with the greatest clearance values generally observed in infants < 20 months of age. Analysis of urinary excretion data from a small subset of our study cohort (i.e., $n = 19$) revealed a significant linear correlation (Figure 2b) between age and linezolid renal clearance ($r^2 = 0.43$, $P = 0.002$). In contrast no correlation was observed between subject age and the nonrenal clearance (estimated as the difference between Cl and Cl_{renal}) for linezolid ($r^2 = 0.10$, $P > 0.05$; Figure 2b). The age-related variability observed in Cl_{renal} did not appear to result from developmental differences in the cumulative urinary excretion of linezolid as the percent of total dose recovered in urine during 24 h did not vary significantly with age ($r^2 = 0.14$, $P > 0.05$). Overall nonrenal clearance accounted for the majority (i.e., ~80%) of total clearance with nonrenal and renal clearance parameter estimates of 131.5 ± 89.6 ml/min and 30.7 ± 9.9 ml/min, respectively. No significant correlation was observed between age and the apparent volume of distribution for linezolid in our study cohort.

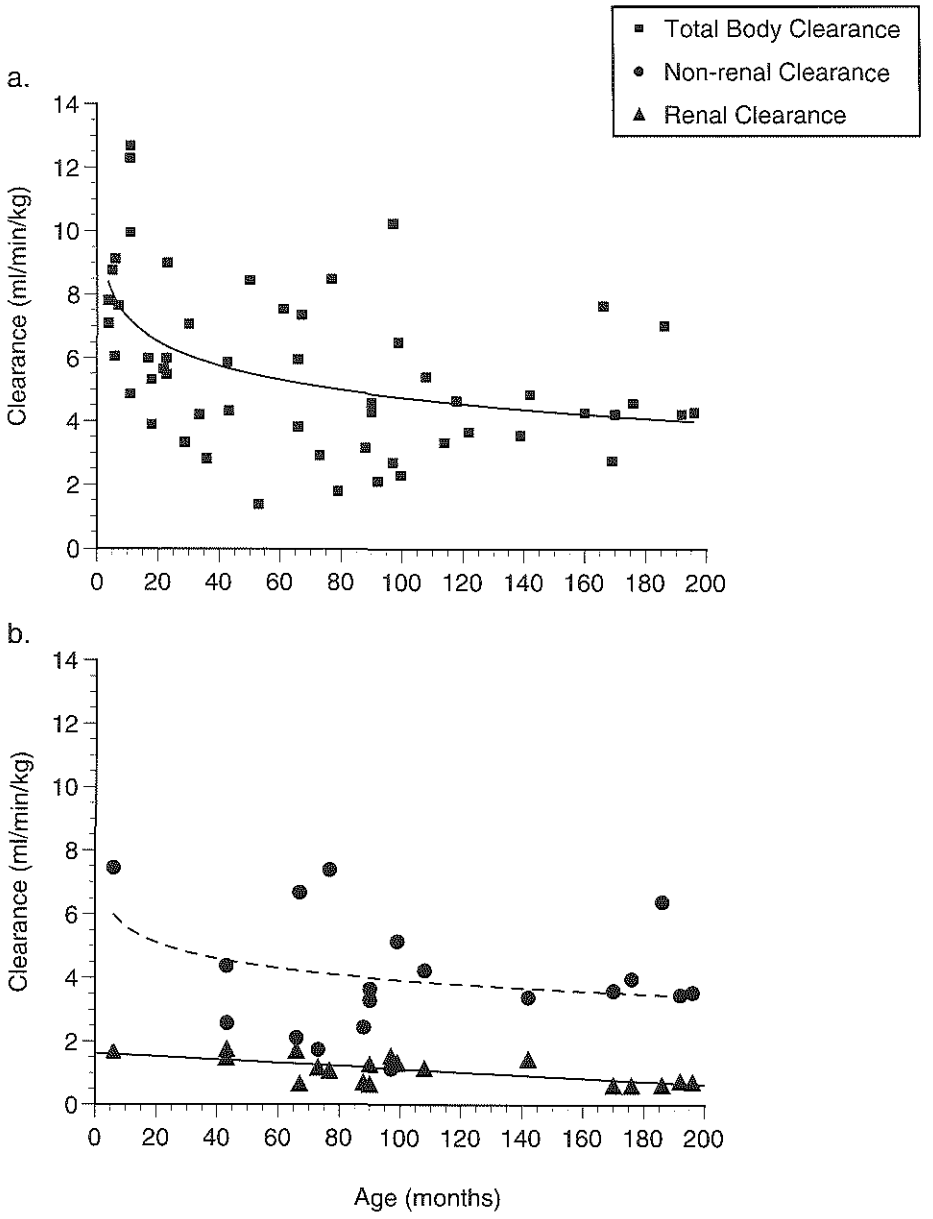

Figure 2 *Correlation between age and both linezolid total body clearance (a, n = 54, r^2 = 0.24, P = 0.004) and renal clearance (b, n = 19; r^2 = 0.43, P = 0.002). An association was observed between the calculated nonrenal clearance for linezolid and age in a subset of the study cohort with complete urine collections (n = 19) despite the lack of a statistically significant correlation.*

Table 2. Individual Pediatric Demographic and Pharmacokinetic Parameters

Variable	Pediatric 1.5 mg/kg	Pediatric 10 mg/kg
No. of subjects	40	14
Age (ys)	5.4 ± 4.9 (0.3-16)†	7.9 ± 4.4 (1.5-16.3)
Weight (kg)	21.8 ± 15.7 (5.3-60)	30.1 ± 16.1 (10.8-63.5)
C_{max} (mg/l)	2.5 ± 0.8 (1.4-4.7)	15.3 ± 4.7 (10.9-28.9)
Dose-normalized C_{max} (mg/l/mg dose)	0.11 ± 0.06 (0.02-0.22)	0.07 ± 0.06 (0.02-0.21)
T_{max} (h)	0.56 ± 0.1 (0.5-1)	0.54 ± 0.07 (0.5-0.75)
$AUC_{0-\infty}$ (mg*hr/l)	5.22 ± 3.18 (2-18)	44.2 ± 17.0 (19.6-79.2)
Dose-normalized AUC (mg*hr/l/mg dose)	0.21 ± 0.13 (0.04-0.61)	0.19 ± 0.11 (0.06-0.37)
Half-life (h)	3.1 ± 1.1 (1.4-5.4)	2.7 ± 0.9 (1.1-4.8)
Plasma clearance (l/hr/kg)	0.36 ± 0.16 (0.08-0.76)	0.26 ± 0.11 (0.13-0.51)
VD_{ss} (l/kg)	0.75 ± 0.18 (0.04-1.1)	0.66 ± 0.18 (0.03-1.0)
C_{12} (mg/l)	0.04 ± 0.08 (0.001-0.46)	0.33 ± 0.51 (0.17-1.76)
C_{24} (mg/l)	0.008 ± 0.016 (0-0.081)	0.07 ± 0.14 (0-0.43)

**mean ± SD*
† Numbers in parentheses, range
C_{max}, peak plasma concentration; T_{max}, time of C_{max}; $AUC_{0-\infty}$, area under the plasma concentration vs. time curve extrapolated to infinity; VD_{ss}, apparent steady state volume of distribution; C_{12}, concentration at 12 h and C_{24}, concentration at 24 h.

Significant age related changes in dose-normalized C_{max} ($r^2 = 0.82$, $P = 0.001$) and $AUC_{0-\infty}$ normalized to dose ($r^2 = 0.54$, $p = 0.001$) were observed and these were consistent with the finding of a significant correlation between age and uncorrected parameter estimates for clearance ($r^2 = 0.62$, $P = 0.001$) and VD_{ss} ($r^2 = 0.84$, $P = 0.001$). A linear relationship between dose and both C_{max} ($r^2 = 0.84$) and AUC ($r^2 = 0.79$) was evident, thereby suggesting dose proportionality over the dosing range evaluated.

4.5 Discussion

Recently, several investigational agents including fourth generation quinolones, synthetic glycopeptides, glycylcyclines, streptogrammins and the oxazolidinones have emerged as potential treatments for drug-resistant pathogens.[17] *In vitro* data suggest that linezolid, the newest of the oxazolidinones, effectively inhibits bacterial growth in a number of Gram-positive organisms, regardless of their resistance phenotype (Table 1). In addition,

preliminary *in vivo* experience with linezolid suggests that this drug may provide a therapeutic alternative in the treatment of human infection.[18,19]

The disposition of linezolid has been relatively well characterized. Animal models suggest extensive distribution of the drug into soft tissue with maximal concentrations relative to plasma of ~ 20% in central nervous system and bone; 40% in eye and testis; 70% in heart, lung, thyroid, skin and muscle; and >150% in liver, kidney, adrenal gland and gastrointestinal tract tissue.[20] In adults, the apparent volume of distribution of linezolid approximates the total body water space.[21] Consequent to known developmental differences in body water spaces relative to body mass, the mean values observed for the apparent volume of distribution for linezolid were slightly larger in children (0.73 ± 0.18 liter/kg) as compared to adults (0.63 ± 0.13 liter/kg).[21] As expected based on the larger apparent volume in children, maximum achievable concentrations (normalized to mg/kg dose administered) were slightly lower in the pediatric subjects (1.62 ± 0.52 mg/l) than in with adults (1.97 ± 0.49 mg/l).[21]

Nonrenal pathways account for ~65% of total body clearance for linezolid with the primary route of elimination arising through oxidation of the morpholine ring which generates inactive metabolites.[22] *In vitro* metabolic screens have revealed that linezolid does not interact with any of the major human cytochromes P-450 as either a substrate or an inhibitor.[23] Thus, age-related changes in the non-renal clearance would not be predicted based on the known ontogeny of these enzymes.

After a single intravenous dose of either 1.5 or 10 mg/kg of linezolid, the apparent bi-phasic nature of the log plasma concentration *vs.* time curve was quite similar between pediatric patients and adults.[21] There was no apparent dose dependence in the pharmacokinetics of linezolid in our pediatric study cohort over a > 6-fold difference in study doses. In contrast, apparent age-associated differences in the plasma clearance of linezolid were apparent. Developmental differences in drug disposition are markedly apparent when linezolid total plasma clearance is compared between adults[21] (0.10 ± 0.03 liter/h/kg) and children (mean values of 0.26 to 0.36 liter/h/kg for the 10- and 1.5-mg/kg dose, respectively). As reflected by the nonlinear correlation between linezolid total body clearance and age, children who were < 40 months of age had the highest clearance values. Further examination of the apparent age dependence in linezolid clearance in a subset of our study cohort revealed that nonrenal clearance of the drug, in contrast to the

renal clearance, most likely attributed to the apparent developmental differences in total body clearance. The reasons for an apparent increase in linezolid clearance in infants and young children are not immediately apparent from the known metabolic profile of the drug given that its biotransformation does not appear to be dependent on P-450 cytochromes. It is possible that age-dependent differences in the activity of other non-cytochrome P-450 isoenzymes capable of catalyzing phase I (e.g., amidases, peptidases, peroxidases) biotransformation reactions[24] are in part responsible for our findings.

Given that estimates of total body exposure are directly influenced by plasma clearance, the calculated AUC (normalized to mg/kg dose administered) was examined in our entire study cohort (n = 54) relative to data from adults.[21] In our pediatric patients the dose-normalized AUC was 3.72 ± 2.04 mg*h/l per mg/kg dose of linezolid; a value that was ~35% of that previously reported for adults (10.51 ± 3.44 mg*h/l per mg/kg dose).[21] As might be expected from the apparent relationship between linezolid total body clearance and age, the dose-normalized AUC for infants (0 to 1 years of age, n = 10, AUC = 2.09 ± 0.64 mg*h/l per mg/kg dose) and young children (1 to 2 years of age, n = 7, AUC = 2.97 ± 0.71 mg*h/l per mg/kg dose) was lower than the mean value for the entire pediatric cohort. Thus in young infants and children, the apparent developmental differences in linezolid disposition impact not only total body and nonrenal clearance but also the level of potential exposure from a given milligram/kg dose of the drug.

In summary linezolid was well-tolerated in pediatric subjects following intravenous administration of single doses of 1.5 and 10 mg/kg. The disposition of linezolid appears to be developmentally dependent with mean values for plasma clearance and apparent volume of distribution of linezolid in infants and children being greater than the corresponding values in adults. Consequently, maximum achievable linezolid plasma concentrations after a given milligram/kg dose of linezolid were slightly lower in infants and children. Also plasma drug concentrations at 12 and 24 h after a single 10-mg/kg dose of linezolid in pediatric patients were below the MIC required to inhibit 90% of susceptible bacteria *in vitro*. Thus pharmacokinetic data obtained in pediatric patients from 0.3 to 16 years of age support an intravenous linezolid dose of 10 mg/kg administered two to three times daily in future trials of drug efficacy and safety. Our pediatric data can not be used to extrapolate a dose for linezolid in term or preterm

neonates where marked developmental differences in drug disposition are commonly observed.

4.6 Acknowledgements

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Chapter 5

Impact of Gestational and Postnatal Age on Linezolid Disposition in Neonates and Young Infants

Based on the article:

Impact of Gestational and Postnatal Age on Linezolid Disposition in Neonates and Young Infants

Kearns GL, Jungbluth GL, Welshman IR, Abdel-Rahman SM, Hopkins NK, Bruss JB, Wu E, van den Anker JN, and The Pediatric Pharmacology Research Unit Network.

5.1 Abstract

Linezolid (LZD) is a marketed, oxazolidinone antibiotic active against Gram-positive bacteria including vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus*. Initial studies demonstrated that plasma clearance (Cl) of LZD in patients 2 to 12 years of age was significantly greater than observed in either adolescent or adult patients. The impact of age on LZD disposition during the first few months of life have not been previously investigated. To fill this information-gap, we characterized LZD disposition following a single, 10.0 mg/kg intravenous dose in 42 infants stratified as follows: Group I ($n = 9$) - gestational age (GA) < 34 weeks, postnatal age (PNA) < 8 days; Group II ($n = 7$) - GA < 34 weeks, PNA 8 days to 12 weeks; Group III ($n = 11$) - GA ≥ 34 weeks, PNA < 8 days and Group IV ($n = 15$) - GA ≥ 34 weeks, PNA 8 days to 12 weeks. LZD was quantitated by a validated HPLC method with tandem mass spectrometric detection from repeated blood samples (0.3 ml each) obtained immediately prior to dosing and at 1.167, 2, 4, 6, and 10 to 12 h after the start of a 60 minute infusion. Pharmacokinetic parameters were determined using standard model dependent techniques. Overall, the values (mean ± SD) for Cl (0.25 ± 0.12 l/h/kg), apparent volume of distribution (VD_{ss} = 0.75 ± 0.19 l/kg) and elimination half life ($T½$ = 2.8 ± 2.1 h) from the study cohort were similar to values reported previously for children and adolescents. However, examination of the LZD pharmacokinetic parameters as a function of age revealed that the Cl of LZD increases rapidly during the first week of postnatal life and that PNA was the most accurate predictor of Cl. Age-stratification based on PNA revealed lower values for Cl (l/h/kg) in those infants < 8 days of age (Group I = 0.12 ± 0.06; Group III = 0.23 ± 0.12) as compared to those 8 days to 12 weeks of age (Group II = 0.31 ± 0.07; Group IV = 0.31 ± 0.10). In contrast to Cl, GA served to be the most useful predictor of VD_{ss}. Evaluation of the pharmacokinetic data in light of the known pharmacodynamics of LZD would appear to support the use dosing regimens for infants and children different from those currently approved for adult indications.

5.2 Introduction

Linezolid, [(S)-N-{{3-{3-Fluoro-4-(4-morpholinyl)phenyl)}-2-oxo-5-oxazolidinyl}methyl}-acetamide] is a currently marketed oxazolidinone antimicrobial

agent that demonstrates considerable potential in the treatment of resistant gram positive organisms which include vancomycin-resistant enterococci (VRE) and methicillin resistant strains of both *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE). The mechanism of action for the oxazolidinones is unique and involves inhibition of early events in protein synthesis, possibly distorting the binding site for the initiation tRNA.[1,2]

The antimicrobial spectrum of linezolid has naturally caused extension of its use into the pediatric population. Kaplan et al.[3] recently reported results from a multicenter study of linezolid given at a dosing regimen of 10 mg/kg every 12 h to treat community-acquired pneumonia in 78 hospitalized children one to 17 years of age. At the end of therapy for infections caused by a diverse spectrum of pathogens (e.g., pencillin-sensitive and resistant strains of *Streptococcus pneumoniae*, Group A *Streptococcus*, MRSA), a composite clinical cure rate of 92.4% was reported. The drug was generally well tolerated with gastrointestinal effects (10.3%), rash (3.8%), mild elevations in ALT (6.4%) and neutropenia (6.4%) being the most commonly reported adverse events during linezolid therapy.

We have previously reported the results from a pharmacokinetic study of linezolid conducted in 58 children and adolescents ranging in age from 3 months to 16 years.[4] This investigation revealed a statistically significant correlation between linezolid total plasma clearance (Cl) and age, with values for both Cl and the apparent volume of distribution (VD_{ss}) that were significantly greater in children as compared to data from adults. Hence, the impact of ontogeny on the disposition of linezolid during the first three months of life is not known.

Given the potential for therapeutic use of linezolid in the neonatal period to treat infections produced by MRSA, MRSE and unusual, highly resistant organisms associated with neonatal meningitis (e.g., *Flavobacterium meningosepticum*),[5] it is critical that the impact of development on linezolid disposition and the potential implications for age-specific dosing be examined. We report herein the results from a multicenter study of linezolid disposition in neonates and young infants conducted to address this information gap.

5.3 Methods

Subjects

Forty-two neonates and young infants were enrolled in this open-label, multicenter clinical investigation. Subjects were eligible for enrollment if they met the following inclusion criteria:

1. postnatal age (PNA) between birth and 12 weeks,
2. body weight greater than the 5th and less than the 95th percentile for age and gender,
3. a documented medical need for antibiotic therapy based upon the presence of suspected or culture-proven infection and
4. the availability of parents and/or legal guardians for the purpose of obtaining informed consent.

Subjects were excluded from participation if any of the following criteria were met:

1. evidence of clinically unstable renal, hepatic, cardiac, hematopoietic and/or pulmonary status as reflected by medical history, physical examination, and/or laboratory tests,
2. a history or evidence of progressive neurologic condition, including intraventricular hemorrhage (Grade III or IV), seizures or any disorder that would impair safe and/or effective completion of all study-related procedures,
3. receipt of an investigational drug administered as part of a clinical trial within 30 days of study drug administration,
4. concomitant treatment with any drug known to induce or inhibit hepatic microsomal enzymes and
5. inability to tolerate required study procedures (e.g., maintaining vascular access sufficient to enable repeated blood sampling).

To facilitate balance with respect to subject age across the study cohort, the study population was divided into four subgroups based upon postnatal age (PNA) and gestational age (GA). These groups are described as follows: Group I - gestational age (GA) < 34 weeks, postnatal age (PNA) < 8 days; Group II - GA < 34 weeks, PNA 8 days to 12 weeks; Group III - GA ≥ 34 weeks, PNA < 8 days and Group IV - GA ≥ 34 weeks, PNA 8 days to 12 weeks. These groupings also enabled comparison of linezolid pharmacokinetic parameters between infants based upon discrete demographic variables.

A medical history, physical examination, and clinical laboratory tests (complete blood count, serum chemistry panel which included serum transaminase, albumin, total protein and creatinine values, and urinalysis) were performed in each subject prior to enrollment and within 24h of study completion. All subjects were enrolled via informed parental consent. The study protocol was approved by the Institutional Review Boards of the participating institutions and the Network Steering Committee of the Pediatric Pharmacology Research Unit Network. The study protocol was conducted under applicable Good Clinical Practice regulations in compliance with all regulatory requirements under the U.S. Food and Drug Administration IND # 55,618.

Study Design

The study was conducted as an open label, single-dose evaluation of linezolid pharmacokinetics and tolerance at one dose level (10 mg/kg). Subjects received linezolid (Zyvox® sterile solution, 2.0 mg/ml, Pharmacia, Kalamazoo, MI) derived from a single lot number as a constant rate infusion delivered into a peripheral vein over 60 minutes by syringe pump with microbore tubing. All subjects were studied during an inpatient stay at the participating institutions where they remained throughout the 12-h post-dose sample collection and observation period.

Sample Collection

Blood samples (0.3 ml each) for determination of linezolid concentration were collected from an indwelling venous catheter placed in either a contralateral extremity or from an existing central venous catheter placed to facilitate medical care unrelated to the pharmacokinetic study at the following times: pre-dose and 1.167, 2, 4, 6 and 10 –12 h following the start of the linezolid infusion. Blood samples were initially placed into polypropylene tubes containing anticoagulant (0.9 mg K_2EDTA; Microtainer®, Becton Dickinson, Franklin Lakes, NJ), were immediately mixed by inversion and were centrifuged (2,500g for 10 minutes at 4°C) to separate plasma which was then removed by manual aspiration, transferred to polypropylene tubes and stored at -70°C within 30 minutes of collection. Vital signs were examined immediately prior to linezolid administration, hourly throughout the study and again with the post-study physical examination and laboratory safety assessment.

Analytical Procedures

Linezolid plasma concentrations were determined using a validated high-performance liquid chromatographic assay.[6] Briefly, plasma samples (100 µl) were spiked with internal standard (^{3}DPNU-100766), buffered with 50 µl of 13 M ammonium acetate, vortexed and extracted with 2 ml of a 75:25 ethylacetate:pentane mixture. The aqueous phase was flash frozen and the organic phase decanted and evaporated to dryness. Samples were reconstituted to 200 µl with 75:25 0.01M ammonium acetate:methanol: and 50 µl was injected onto a C_{18} analytical column (2 x 150 mm, 5µm). The eluate was monitored using atmospheric pressure chemical ionization interface to a triple-quadrapole mass spectrometer (LC/MS/MS) that was operating in a positive selective ion monitoring mode. An eight point standard curve using the ratio of active compound to internal standard was prepared daily and was used to calculate all plasma linezolid concentrations. The limit of quantitation was established at 1 ng/ml for the plasma assay. The analytical method demonstrated linearity at plasma linezolid concentrations ranging from 1 to 250 ng/ml (r^2>0.998). Inter-day and intra-day assay variability was consistently $\leq$ 6.0% and 5.0%, respectively for linezolid plasma concentrations within the range of linearity. All assays were performed by an independent laboratory (AvTech Laboratories, Inc., Kalamazoo, MI). The mean linezolid concentrations from the analysis of duplicate samples was reported and used to accomplish the pharmacokinetic and statistical analyses.

Pharmacokinetic and Statistical Analysis

Pharmacokinetic and statistical analyses were conducted using subroutines contained in the Kinetica® software package (version 4.0, InnaPhase Co., Philadelphia, PA) and SPSS version 9.0 (SPSS, Chicago, IL). Plasma drug concentration versus time data were curve fit using a peeling algorithm to generate initial polyexponential parameter estimates. Final parameter estimates were determined from an iterative, nonlinear weighted least squares regression algorithm with reciprocal (i.e., $1/y^2$) weighting. All pharmacokinetic parameters were derived using a model dependent approach with final model selection based upon assessment of goodness-of-fit criteria. The area under the plasma concentration versus time curve from 0 to 1 2h post-dose ($AUC_{0\text{-}12}$) was determined using the mixed log-linear rule. Extrapolation of the AUC to infinity ($AUC_{0\text{-}\infty}$) was calculated by summation of $AUC_{0\text{-}12} + Cp_{12}/\lambda_z$, where Cp_{12} represented the plasma concentration at 12h post-dose predicted from the fitted apparent terminal elimination phase and λ_z is the

apparent terminal elimination rate constant. Total body clearance (Cl) and steady state volume of distribution (VD_{ss}) were calculated from the $AUC_{0-\infty}$.

Linezolid pharmacokinetic parameters for the study cohort were examined using standard descriptive statisticals. The Wilk-Shapiro test was used to assess the homogeneity of variance for the pharmacokinetic parameters. Analysis of variance was used to assess potential differences in pharmacokinetic parameters between the four subject groups, followed by application of a parametric post-hoc test (Tukey's A) to determine where inter-group differences occurred. Comparison of linezolid pharmacokinetic parameters between patients with PNA <8 days and ≥ 8 days was conducted using a two-tailed, unpaired Student's t-test. Examination of the data for possible age dependence in the linezolid pharmacokinetic parameters (e.g., λ_z, Cl, VDss, $AUC_{0-\infty}$, Cmax) was undertaken using linear and non-linear regression models. Binary logistic regression was used to evaluate the relationship between demographic variables and predicted pharmacodynamic parameters. The significance limit accepted for all statistical analyses was $\alpha = 0.05$.

5.4 Results

Forty-two subjects (28 male, 6 African American, 1 mixed race) ranging in PNA from 1 to 79 days (19.8 ± 21.5) and GA from 25 to 40 weeks (34.7 ± 3.8) completed this pharmacokinetic investigation. Both height (47.9 ± 6.95, 32 to 73 cm) and weight (2.78 ± 1.24, 0.74 to 6.2 kg) were appropriate for postconceptional age in all subjects. Linezolid was well tolerated in the pediatric subjects with only mild adverse events reported (mild and transient erythema at catheter site) in association with the study procedures. No serious adverse events were described in the subject cohort during the period of study.

The mean (+ SD) and median plasma linezolid concentration versus time data over the 12-hour post-dose period are illustrated in Figure 1a and the individual subject data are provided in Figure 1b. As illustrated by Figures 2a and 2b, Cl and λ_z increased dramatically as a function of postnatal age through the first week of life after which both parameters remained relatively constant through 79 days. A segmented linear model proved the best predictor for both Cl ($r^2 = 0.407$, $P < 0.001$) and λ_z ($r^2 = 0.568$, $P < 0.001$) with a slight but significant improvement in correlation for λ_z when gestational age was

introduced into the model ($r^2 = 0.623$, $P < 0.001$). The resultant equation for both parameters is provided as follows:

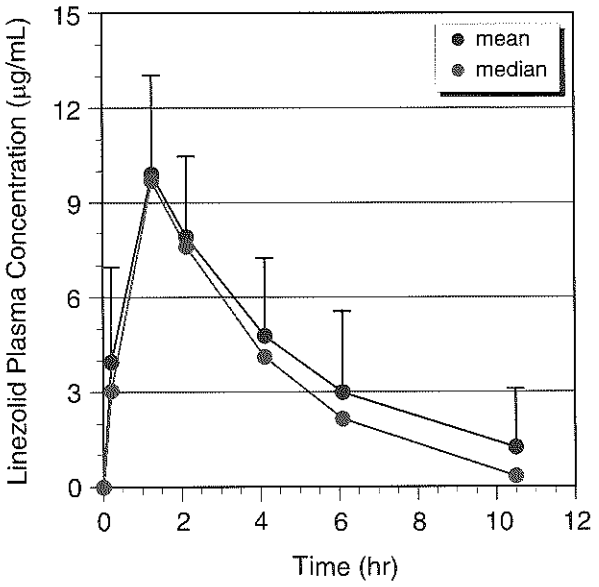
λ_z (1/ h) =	(0.04922*PNA) + (0.01054*GA) – 0.2796	if PNA $\leq$ 8 days
λ_z (1/ h) =	(0.01054*GA) + 0.0649	if PNA > 8 days
Cl (l/ h/kg) =	(0.03471*PNA) + 0.06603	if PNA $\leq$ 8 days
Cl (l/ h/kg) =	0.309	if PNA > 8 days

where PNA is in represented in days and GA is in weeks.

When compared between the four subject groups, stratified by PNA and GA, statistically significant differences ($P < 0.05$) were found for estimates of Cl (Table 1) as well as other parameters indicative of elimination (e.g. λ_z, T½). Although the principal differences observed in Cl were seen between neonates with a PNA< 8 days and GA< 34 weeks (Group I) and infants with a PNA $\geq$ 8 days irrespective of GA (Groups II and IV), a statistically significant difference could also be observed when the data were combined by PNA alone, with Cl values of 0.18 ± 0.11 l/ h/kg and 0.31 ± 0.09 l/ h/kg for infants < 8 days ($n = 20$) and > 8 days ($n = 22$), respectively ($P < 0.001$). As expected, the differences observed between group with respect to T½ and λ_z are consistent with the observations for Cl. In addition to age, gender differences for linezolid Cl were also evaluated. No statistically significant difference was apparent when linezolid Cl was compared between females (n = 14, 0.29 ± 0.10 l/ h/kg) and males ($n = 28$, 0.23 ± 0.12 l/ h/kg).

In contrast to Cl, the VD_{ss} of linezolid was most closely associated with GA ($r^2 = 0.24$, $P = 0.001$) in an inverse fashion (Figure 2d) and could be described according to the following equation: $VD_{ss} = 1.622 – (0.025*GA)$. Although an age-dependent trend was observed for VD_{ss} in young infants, statistically significant differences were not found for this pharmacokinetic parameter when compared between the age-stratified subgroups (Table 1).

a.



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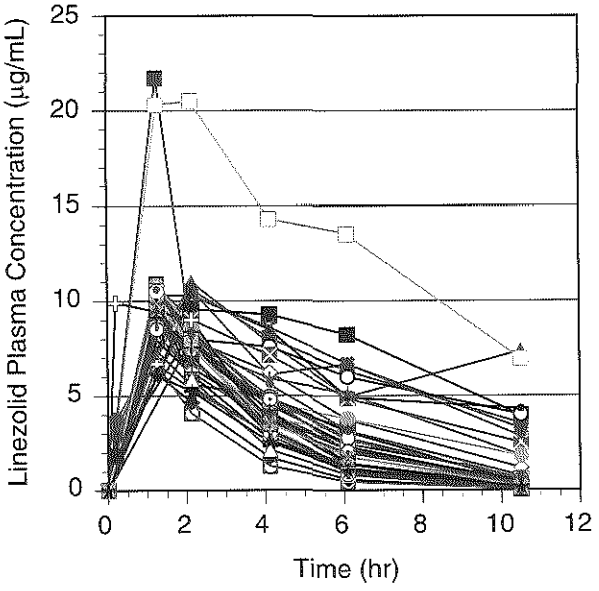


Figure 1: *(a) Mean (+ SD) and median and (b) individual plasma linezolid concentration vs. time data in 42 neonates and young infants*

Table 1 Summary of Linezolid Pharmacokinetic Parameters Overall and by Group from Neonates and Young Infants ($n = 42$)

PARAMETER	OVERALL	GROUP I	GROUP II	GROUP III	GROUP IV
		PNA<8 d, GA<34 wk	PNA≥8 d, GA<34 wk	PNA<8 d, GA≥34 wk	PNA≥8 d, GA≥34 wk
	$n = 42$	$n = 9$	$n = 7$	$n = 11$	$n = 15$
C_{max} calc (mcg/ml)	11.93 ± 3.23 (7.15-22.17)	12.7 ± 3.9 (9.6-22.2)	9.8 ± 1.5 (8.3-12.4)	11.5 ± 2.6 (8.0-18.3)	12.8 ± 3.6 (7.2-21.6)
T_{max} calc (h)	1.0 ± 0.1 (1.0-1.4)	1.1 ± 0.1 (1.0-1.4)	1.0 ± 0.0 (1.0-1.1)	1.0 ± 0.0 (1.0-1.1)	1.0 ± 0.1 (1.0-1.3)
$AUC_{0-\infty}$ (mg*h/l) *†‡	54.92 ± 39.33 (16.89-190.70)	108.10 ± 50.59 (41.38-190.70)	33.98 ± 8.01 (25.78-44.00)	53.41 ± 25.19 (18.98-102.54)	33.88 ± 8.54 (16.89-49.52)
λ_z (1/h) *†‡¥§	0.345 ± 0.161 (0.071-0.578)	0.150 ± 0.068 (0.070-0.290)	0.357 ± 0.070 (0.250-0.460)	0.304 ± 0.143 (0.110-0.550)	0.488 ± 0.092 (0.290-0.580)
T_{half} (h) *†‡	2.8 ± 2.1 (1.2-9.8)	5.6 ± 2.5 (2.4-9.8)	2.0 ± 0.4 (1.5-2.8)	2.9 ± 1.6 (1.3-6.1)	1.5 ± 0.4 (1.2-2.4)
Cl (l/h/kg) *‡	0.25 ± 0.12 (0.05-0.59)	0.12 ± 0.06 (0.05-0.24)	0.31 ± 0.07 (0.23-0.39)	0.23 ± 0.12 (0.09-0.53)	0.31 ± 0.10 (0.20-0.59)
VD_{ss} (l/kg)	0.75 ± 0.19 (0.35-1.08)	0.81 ± 0.19 (0.43-1.05)	0.87 ± 0.13 (0.67-1.01)	0.78 ± 0.15 (0.45-0.96)	0.65 ± 0.21 (0.35-1.08)

Data are reported as mean ± sd (range)
PNA, postnatal age; GA, gestational age; wk, weeks; d, days; C_{max}, peak plasma concentration; T_{max}, time of C_{max}; AUC_{0-inf}, area under the plasma concentration vs. time curve extrapolated to infinity; λz, apparent terminal elimination rate constant; T_{half}, apparent terminal elimination half life; Cl, apparent total plasma clearance and VDss, apparent steady state volume of distribution.
** denotes significant difference between Group I vs. Group II*
† denotes significant difference between Group I vs. Group III
‡ denotes significant difference between Group I vs. Group IV
¥ denotes significant difference between Group II vs. Group IV
§ denotes significant difference between Group III vs. Group IV

Maximum linezolid plasma concentrations (C_{max}) demonstrated only a moderate degree of variability (approximately 3-fold) across the subjects evaluated with C_{max} increasing as a function of GA ($r^2 = 0.11$, $p = 0.031$, data not shown). The calculated linezolid plasma concentrations at 8 and 12h post-dose demonstrated a more marked degree of inter-individual variability with mean ± SD (range) values of 1.89 ± 2.17 (0.15 to 9.34) and 0.96 ± 1.50 (0.02 to 5.7) mg/l at 8 and 12h post-dose, respectively. The fact that GA was a better predictor of VD_{ss} as compared to C_{max} suggests that the variability observed in C_{max} is likely a function of known age-related changes in body composition[7] that appear to be reflected by the VD_{ss}.

Total body exposure, as reflected by $AUC_{0\rightarrow\infty}$, demonstrated a marked degree of intersubject variability and was correlated with PNA during the first week of life (Figure 2c). When stratified by PNA, a statistically significant difference ($P < 0.05$) was found for $AUC_{0\rightarrow\infty}$ (Table 1). Consistent with the linezolid Cl data, the age-associated differences observed for $AUC_{0\rightarrow\infty}$ appeared to be explained by a slower rate of linezolid elimination in the youngest children. When fit to a segmented linear model, approximately 38% of the variability could be accounted for by PNA ($r^2 = 0.377$, $P < 0.001$) with no additional improvement by the addition of GA. Given that postnatal age is a stronger predictor of Cl and λ_z than of $AUC_{0\rightarrow\infty}$, it would appear that age-dependent effects on elimination, in the absence of other factors, likely account for the variability observed in $AUC_{0\rightarrow\infty}$.

In two subjects a marked increase in $AUC_{0\rightarrow\infty}$ was observed following the single 10 mg/kg dose (Figure 1b). Inspection of the individual pharmacokinetic parameters for these subjects revealed an apparent VDss on the low end of the range for all observed values in the study cohort (i.e., 0.35 and 0.4 l/kg, respectively). This reduction in VD_{ss}, as opposed to a reduction in elimination, likely accounted for the disparity in $AUC_{0\rightarrow\infty}$ given that the corresponding values of λ_z in these two subjects were not markedly different from the population mean (i.e., 0.34 1/h).

As time above the minimum inhibitory concentration (MIC) appears to be the putative pharmacodynamic parameter of importance in determining linezolid efficacy,[1] an exploratory analysis that examined the pharmacokinetic profile in the neonate in relation to the pharmacodynamic profile was conducted. The pharmacodynamic profile (i.e. time above MIC) was simulated for each child based on his/her individual pharmacokinetic data.

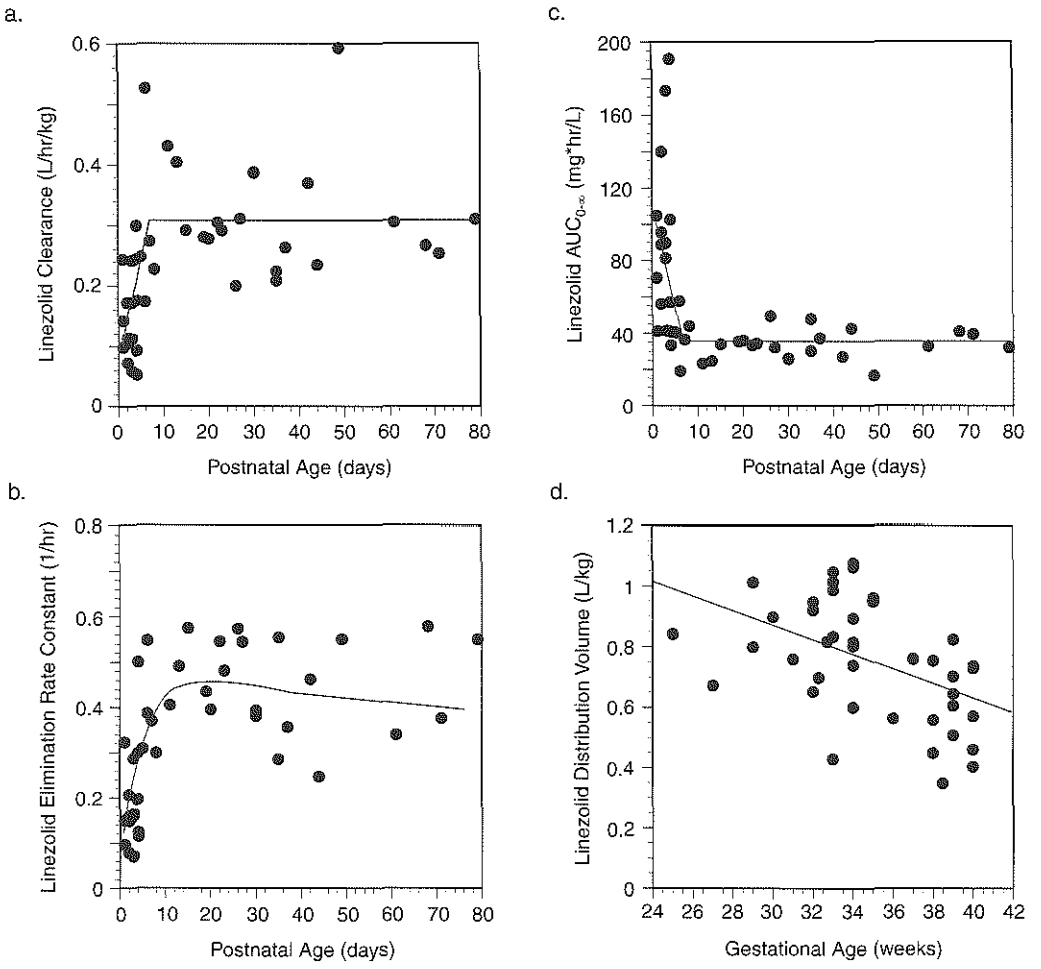


Figure 2: *Association between (a) linezolid plasma clearance (r^2=0.407, P < 0.001) (b) apparent terminal elimination rate constant (r^2=0.568, p<0.001) and (c) AUC (r^2=0.377, P < 0.001) with postnatal age, and (d) apparent steady state volume of distribution (r^2=0.244, P = 0.001) with gestational age.*

The calculated mean ± SD (range) for percent of the dosing interval where plasma linezolid concentrations were projected to remain above arbitrary MIC values of 1, 2 and 4 mg/l were 82 ± 19 (45-100), 67 ± 24 (29-100) and 48 ± 28 (13-100)%, respectively for an 8-hour dosing interval and 64 ± 25 (30-100), 52 ± 28 (19-100) and 35 ± 25 (9-100)%, respectively for a 12-hour dosing interval. Logistic regression was performed to generate the probability curves that describe the likelihood of exceeding the MIC for 40% of a

dosing interval based on previous investigations suggesting that this approximates the pharmacodynamic optima for linezolid.[8] A linezolid dose of 10 mg/kg, dosing intervals of 8 and 12h and MIC values for potential pathogens of 1, 2 and 4 mg/l were evaluated with the resultant curves depicted in Figure 3.

5.5 Discussion

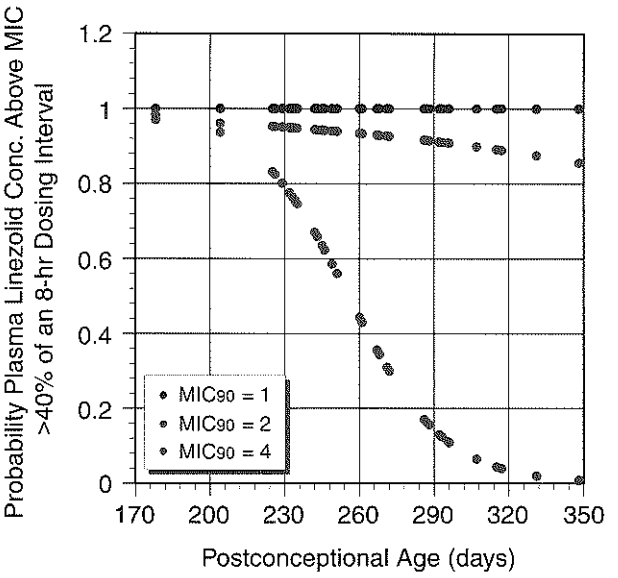
Drug resistant strains of *Enterococci, Streptococci* and *Staphylococci* represent a growing concern among clinicians who treat both adults and children. Within select pediatric populations, rates of colonization with VRE have been reported as high as 40% with resultant clinical infection arising in as many as 10% of those colonized.[9-11] These organisms pose a particular threat to newborns infected with *Enterococci* which is now estimated to be the second leading cause of nosocomial infection and the third leading cause of late onset sepsis in neonatal intensive care units.[12] As well, neonatal infections caused by MRSA and MRSE [12] and the increasing prevalence of intermediate and high level pencillin resistance for *S. pneumoniae* [13,14] provide a potential pharmacotherapeutic niche for linezolid use in neonates and young infants. Given the relationship between the pharmacokinetics and pharmacodynamics for antimicrobials and the previous observation of age-dependent disposition for linezolid,[4] it was both prudent and necessary to examine linezolid pharmacokinetics in the youngest of the potential recipients of this drug.

The disposition of linezolid has been well characterized in adults,[15-19] and recently, in children and adolescents.[4] The plasma concentration vs. time profile for linezolid observed in our cohort of neonates and young infants (Figure 1) was similar to that we observed previously[4] in children and adolescents who also received a single 10.0 mg/kg intravenous dose. The range of apparent C_{max} values in the neonates and infants of this study (11.93 ± 3.23, 7.2 to 22.2 mg/l) was similar to the range for C_{max} values reported previously for pediatric patients (15.3 ± 4.7, 10.9 to 28.9 mg/l). This finding is likely a result of comparable values for the VD_{ss} of linezolid between the present (0.75 ± 0.19 L/kg) and previous (0.66 ± 0.18 l/kg) study.[4] While we did find a significant inverse linear correlation between GA and VD_{ss} in our study cohort, considerable variability in this particular pharmacokinetic parameter likely precluded the demonstration of significant differences between subgroups when stratified for PNA and GA (Table 1). Consequent to known developmental differences in body water spaces relative to body mass,[7] a

distribution volume for linezolid that approximates the total body water space,[1] and values for VD_{ss} in the pediatric population[4] that exceed those reported in adults[15,16,19] the relationship between VD_{ss} and GA found in our subjects (Figure 2d) were expected. The slightly larger VD_{ss} for linezolid observed in young neonates and infants explains the average C_{max} values that were slightly smaller than those previously reported for children and adolescents receiving the same weight-based dose, as noted above.[4]As was the case for VD_{ss}, the mean Cl for linezolid in neonates and infants (0.25 ± 0.12 l/h/kg) was virtually identical to the value (0.26 ± 0.11 l/h/kg) determined from our previous pediatric pharmacokinetic study.[4] However, the disparity in Cl values becomes apparent when neonates < 8 days PNA (0.18 ± 0.11 l/h/kg) are compared to infants 8 days to 12 weeks PNA (0.31 ± 0.09 l/h/kg) and older children and adolescents (0.36 ± 0.16 l/h/kg for 1.5 mg/kg linezolid dose; 0.26 ± 0.11 l/h/kg for 10 mg/kg dose).[4] Clearly, a dramatic increase in linezolid Cl occurs during the first week of life (Figure 2a). The most likely explanation for this developmental difference in linezolid Cl resides with the impact of ontogeny on the drug's biotransformation.

In man, non-renal pathways account for approximately 65% of total body clearance for linezolid. The drug is biotransformed into two inactive, morpholine ring-oxidized metabolites that are excreted in the urine and feces. A small fraction of the dose is metabolized via amidase and deamination/reduction pathways to a third inactive metabolite.[15] *In vitro* metabolic screens have revealed that linezolid is not a substrate for any of the major human cytochromes P450.[8] Thus, the apparent age related changes in the non-renal clearance of linezolid previously reported[4] would not be predicted based on the known ontogeny of these enzymes.[7] In contrast, it is possible that during the first week of life, developmentally associated reductions in the activity of enzymes responsible for formation of the lactam metabolite and/or the deaminated metabolite could well reduce the rate of linezolid biotransformation. As well, normal developmental reductions in both glomerular filtration and/or active tubular secretion that occur in the first days and weeks of extrauterine life[7] could impair the normal renal clearance of unchanged linezolid and its major metabolites, thus producing an overall reduction in the apparent total plasma clearance. In the absence of data that confirm a developmental dependence in amidase activity and renal excretion (or formation clearance) of the major linezolid metabolites in our study cohort, the aforementioned assertions pertaining to the reasons for markedly reduced Cl during the first week of life remain speculative.

a.



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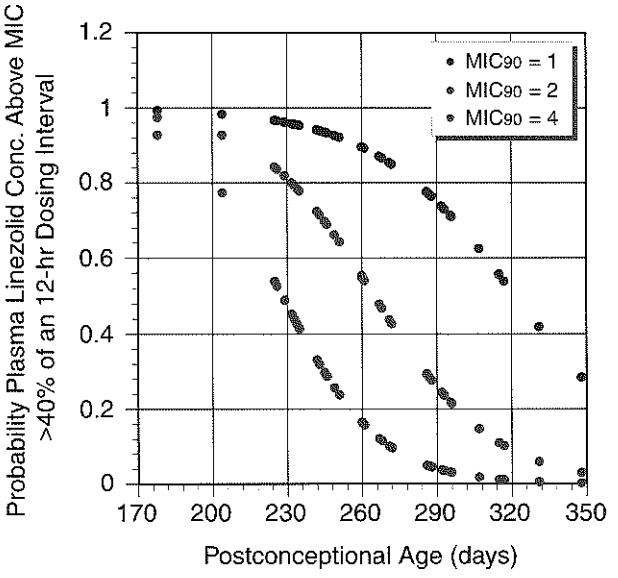

Figure 3: *Binary logistic regression examining the probability of producing linezolid plasma concentrations exceeding the pharmacodynamic optima (i.e., concentrations above the MIC for > 40% of a given dosing interval) for infants receiving a 10 mg/kg with an every (a) 8 h and (b) every 12 h dosing interval.*

A recent study of linezolid in adults demonstrated a mean plasma clearance for the drug in females following oral administration that was 37% less than that for males when values were not corrected for total body weight and 20% when corrected for weight.[19] This prompted us to examine Cl data within our study cohort for a possible sex difference in linezolid PK. As denoted above, no statistically significant difference was apparent when linezolid Cl was compared between females and males. Data from a sufficient number of subjects less than eight days of PNA were not available to afford a meaningful comparison of potential sex-associated differences in Cl during the first week of life. Despite this limitation, these findings suggest that the plasma clearance of linezolid in neonates and young infants does not demonstrate significant sex differences.

Previous pediatric pharmacokinetic data for linezolid were examined in association with the predicted pharmacodynamic properties of the drug by assessing the degree of systemic exposure (reflected by AUC) observed with a 12-hour regimen relative to the sensitivity of potential pathogens.[4] These data appeared to provide support for investigation of an every eight-hour dosing interval for pediatric patients in order to "compensate" for developmental differences in linezolid Cl and thereby, produce a near equivalent exposure to that observed in adults administered the drug on an every 12h dosing schedule. With the possible exception of neonates less than 8 days of age, the similarity in linezolid Cl between infants and older children as denoted above would suggest that a 10 mg/kg every 12h dosing regimen for the drug could potentially result in under-dosing of some infants. This supposition was evaluated in the current study by exploring the pharmacokinetic-pharmacodynamic relationship for linezolid using patient-specific pharmacokinetic parameter estimates, average MIC values and established pharmacodynamic optima (i.e., plasma concentration above MIC for > 40% of a dosing interval) for linezolid. The binary logistic regression produced from these data (Figure 3) clearly illustrates that for susceptible pathogens with a linezolid MIC of $\leq$ 2 mg/l, a 10 mg/kg every 12h dosing regimen should portend therapeutic efficacy for the majority of infants who have a postconceptional age of 230 days or less. This may not be the case for susceptible pathogens with an MIC > 2 mg/l and/or infants who have a high linezolid plasma clearance (i.e., $\geq$ 2 standard deviations above the mean). In this particular instance, a 10 mg/kg every 8-hour dosing regimen (Figure 3) would appear to have a greater chance of producing sufficient systemic exposure necessary to translate into therapeutic efficacy.

5.6 Conclusions

The single intravenous 10 mg/kg dose of linezolid was well tolerated by all neonates and infants. This dose produced a plasma concentration vs. time profile similar to that observed in older children and adolescents in infants greater than one week of age. In contrast to these neonates where linezolid plasma clearance was comparable to values previously observed for older children, the plasma clearance during the first week of life was markedly (i.e., approximately 50%) lower but "matured" rapidly (i.e., by 8 days of life). Accordingly, the disposition of linezolid appears to be developmentally dependent with alterations near the time of birth, as observed herein, and prior to adulthood, as reported previously.[4] Examination of the pharmacokinetic-pharmacodynamic interface for linezolid would appear to support a 10 mg/kg every 8h dosing regimen for the majority of infants and children to achieve a degree of systemic exposure similar to that observed in adults with conventional dosing (i.e., 10 mg/kg every 12h) and thus, minimize any potential compromise of efficacy in treating infections produced by susceptible bacterial pathogens.

5.7 Acknowledgements

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Chapter 6

Cytochrome P450 3A: Ontogeny and Drug Disposition

Based on the article:
Cytochrome P450 3A: Ontogeny and Drug Disposition
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6.1 Abstract

Maturation of organ systems during fetal life and childhood exerts a profound effect on drug disposition. The maturation of drug-metabolizing enzymes is probably the predominant factor accounting for age-associated changes in non-renal drug clearance. The group of drug-metabolizing enzymes most studied is the cytochrome P-450 (CYP) superfamily. The CYP3A subfamily is the most abundant group of CYP enzymes in the liver and consists of at least 3 isoforms: CYP3A4 , 3A5 and 3A7. Many drugs are mainly metabolized by the CYP3A subfamily. Therefore, maturational changes in CYP3A ontogeny may impact on the pharmacokinetics of many of these drugs.

CYP3A4 is the most abundantly expressed CYP and accounts for approximately 30-40 % of the total CYP content in human adult liver and small intestine. CYP3A5 is 83% homologous to CYP3A4, is expressed at a much lower level than CYP3A4 in the liver, but is the main CYP3A isoform in the kidney. CYP3A7 is the major CYP isoform detected in human embryonic, fetal and newborn liver, but is also detected in adult liver, although at a much lower level than CYP3A4. Substrate specificity for the individual isoforms has not been fully elucidated. Because of large interindividual differences in CYP3A4 and CYP3A5 expression and activity, genetic polymorphisms have been suggested. However, although some gene mutations have been identified, the impact of these mutations on pharmacokinetics of CYP3A substrates has to be established.

Ontogeny of CYP3A activity has been studied in *vitro* and in *vivo*. CYP3A7 activity is high during embryonic and fetal life and decreases rapidly during the first week of life. Conversely, CYP3A4 is very low before birth, but increases rapidly thereafter reaching 50% of adult levels between 6 and 12 months of age. During infancy, CYP3A4 activity appears to be slightly higher than in adults. Large interindividual variation in CYP3A5 expression and activity was observed during all stages of development, but no apparent developmental pattern of CYP3A5 activity has been identified to date. Profound changes occur in the activity of CYP3A4 during all stages of development. These changes have, in many instances, proven to be of clinical significance when treatment involves drugs that are substrates, inhibitors or inducers of CYP3A.

6.2 Introduction

Maturation of organ systems during fetal life and in the neonatal and paediatric period exerts a profound effect on drug disposition. From fetal life through adolescence, dramatic changes in pharmacokinetics occur. Accordingly, effective and safe drug therapy in neonates, infants and children requires a thorough understanding of human developmental biology and of the dynamic ontogeny of drug absorption, distribution, metabolism and drug excretion.[1]

Drug metabolism is one of the major determinants of drug clearance in a given individual. Although the relative size of the liver and of hepatic blood flow may affect the rate of drug metabolism during development, the maturation of the drug-metabolising enzymes is probably the predominant factor accounting for age-associated changes in nonrenal drug clearance.[2]

The group of drug-metabolizing enzymes most studied is the cytochromes P450 (CYP). The CYP3A subfamily, the most abundant subfamily of the CYP isoforms in the liver, consists of at least four isoforms: CYP3A4, 3A5, 3A7 and CYP3A43.[3,4] Recently, developmental changes in CYP3A expression and catalytic activity have been studied,[5-9] demonstrating a different ontogenic pattern for the individual CYP3A isoforms. Accordingly, the pharmacokinetics of CYP3A substrates may change as a function of developmental changes in CYP3A activity.[10]

In view of the profound age-related differences in CYP3A activity, physicians who prescribe drugs for neonates, infants and children should be aware of the interindividual differences that often result from developmental patterns of enzyme expression and activity. The goal of this review is to provide the practitioners with insight into the developmental aspects of CYP3A activity and their possible impact on drug therapy.

6.3 Structure and function of Cytochrome P450 (CYP)3A

The CYP family

The CYPs represent a superfamily of heme-containing proteins that catalyze the

metabolism of many lipophilic endogenous substances and exogenous substrates. The biological and pharmacological relevance of this gene family has been the subject of several recent publications.[11-13]

The nomenclature for the P450 superfamily is based on divergent evolution of the genes. CYPs, that share at least 40% homology are grouped in a family denoted by an Arabic number (e.g. CYP3). A subfamily, indicated by a letter, represents highly related genes (e.g. CYP3A). Another Arabic numeral (e.g. CYP3A4) sequentially numbers the individual genes. In humans, 17 CYP gene families have been described to date; the first 3 families are largely involved in the biotransformation of pharmaceuticals and xenobiotics, wheras the other gene families represent genes responsible for the biotransformation of endogenous compounds.[4] The most abundant and most involved in drug metabolism are the isoforms of the CYP3A subfamily.[11]

The CYP3A subfamily

The CYP isoforms belonging to the 3A subfamily account for the majority of drug-metabolising enzymes present in adult human liver and intestine.[14-17] The CYP3A subfamily consists of at least 3 functional genes: CYP3A4, CYP3A5 and CYP3A7,[4] which are located on chromosome 7.[18] The enzymes compromising the CYP3A family share at least 85% amino acid sequence homology. However, they have been shown to differ substantially in substrate specificity and expression.[11] The existence of many additional CYP3A genes is not likely, since a single CYP3A gene is approximately 30 kb in length and only 90 kb of human genomic DNA hybridizes with CYP3A complementary DNA under low-stringency conditions.[19]

CYP3A4

CYP3A4 is the most abundantly expressed CYP in both the human liver and in the small intestine and accounts for approximately 30%-40% of total CYP content in both liver and intestine.[14,20] In adult human liver, CYP3A immunoreactivity was detected in midzonal and centrilobular regions,[21] whereas intestinal CYP3A occurs in the enterocytes lining the lumen of the small intestine.[14,20] Inoue et al.[18] assigned the CYP3A4 gene to chromosome 7 at band q22.1. The gene is divided in 13 exons and 12 introns with a length of approximately 27 kb.[22]

The catalytic properties of CYP3A4 have been studied extensively using expressed

enzyme, immunoinhibition experiments and the correlation of metabolic activity and immmunoreactive CYP3A4 content in microsomal samples.[11] The list of exogenous substrates of CYP3A4 is growing fast (table I) and includes a large number of therapeutically important drugs such as erythromycin, midazolam, cyclosporin, lidocaine (lignocaine) and nifedipine.[10] Endogenous substrates include steroids as testosterone, cortisol, progesterone, androstanediol, dehydroepiandrosterone 3-sulfate (DHEA-S) and estradiol.[11] CYP3A4 also metabolizes procarcinogens as sterigmatocystin and aflatoxin B1.[23]

CYP3A5

CYP3A5 is 83% homologous to CYP3A4 and is also found in hepatic tissue, although at a lower level than CYP3A4. Large interindividual differences for CYP3A5 expression are found. Initially, CYP3A5 mRNA and protein were detected in 10 to 30% of adult human liver samples.[15,26-29] More recently, however, Jouanaidi et al.[30] detected CYP3A5 mRNA in all livers and CYP3A5 protein in 74% (14 out of 19) of all livers examined . This apparent difference in expression of CYP3A5 may be explained by the use of more sensitive analytical methods.[30] It should also be noted that expression of CYP3A5 mRNA differed widely between individuals, ranging from little CYP3A5 mRNA in some individuals to CYP3A5 being the only CYP3A expressed in others.[30]

The substrate specificity of CYP3A5 appears to be similar to that of CYP3A4. However, some differences in catalytic properties have been found. In a reconstituted system, the formation rate of 1-hydroxy-midazolam is considerably higher with CYP3A5 than with CYP3A4. In contrast, the rate of formation of 4-hydroxymidazolam with CYP3A4 and CYP3A5 is similar.[31] No CYP3A5 catalytic activity was found towards quinidine, 17α-ethinylestradiol and aflatoxins,[32] all substrates for CYP3A4. However, Gillam et al.[34] did find considerable catalytic activity of CYP3A5 towards both erythromycin (about 6 times higher when compared to CYP3A4) and ethylmorphine. Interestingly, Gorski et al.[31] found a much better correlation between midazolam hydroxylation and erythromycin *N*-demethylation when livers containing both CYP3A4 and CYP3A5 were excluded from analysis, a finding which supports the different isoform specificity for these drugs. Wrighton et al.[32] also reported that erythromycin is not metabolized by CYP3A5. The formation of carbamazepine- 10,11-epoxide from carbamazepine, and 2-sulphamoylacetylphenol (SMAP) from zonisamide catalyzed by CYP3A5 was approximately 33% and 10% , respectively, as compared with CYP3A4 activity.[34]

Table 1 Important substrates for cytochrome P450 (CYP) 3A[24,25]

Drugs		
Antihistamines	*Anti-fungals*	*Anesthesia/analgetics*
Astemizole	Ketoconazole	Alfentanil
Mizostaline	Miconazole	Fentanyl
Terfenadine	*Immunosuppressants*	Lidocaine
Anti reflux	Cyclosporin (M17 formation)	Ethylmorphine
Cisapride	Cyclosporin (M1 formation)	*Antihypertensives*
Anti-emetic	Tacrolimus (FK-506)	Amlodipine
Ondansetron	*Chemotherapeutics*	Felodipine
Anticonvulsants	Busulfan	Isradipine
Carbamazepine	Doxorubicin	Nicardipine
Clonazepam	Etoposide	Nifedipine
Ethoxisumide	Tamoxifen (also 2D6)	*Anti-arrhythmics*
Zonisamide	Vinblastine	Verapamil
Anti-HIV	Vincristine	Quinidine
Indinavir	*Benzodiazepines*	*Antidepressants*
Ritonavir	Alprazolam	Imipramine
Saquinavir	Diazepam (minor)	Nafzadone
Antimicrobials	Midazolam (1-hydroxy formation)	Sertraline
Clindamycin	Midazolam (4-hydroxy formation)	*Miscellaneous*
Erythromycin	Temazepam	Dextromethorphan
Rifampicin (rifampin)	Triazolam	
Xenobiotics		
Aflatoxin B1	Benzopyrene activation	Sterigmatocystin
Benzphetamine	Heterocyclic amines	
Endogenous substrates		
Androstanedione (6β-hydroxylation)	Estradiol	Testosterone (2β-hydroxylation)
Cortisol (6β-hydroxylation)	17-α-ethinylestradiol	Testosterone (6β-hydroxylation)
Dehydroepiandrosterone	Progesterone (6β-hydoxylation)	Testosterone (15β-hydroxylation)
Dehydroepiandrosterone sulfate		

CYP3A5 actively metabolizes estradiol, DHEA-S and cortisol.[32] However, Ohmori et al.[34] did not find any activity towards DHEA-S by heterologously expressed CYP3A5. The 6β-hydroxylation of testosterone by CYP3A5 occurred at about 10% of CYP3A4 activity in one study,[34] but was similar in another.[33]

In summary, the specificity of CYP3A4 and 3A5 for biotransformation of many substrates appears to be similar, although the extent and rate of metabolic conversion by the individual isoforms may be quite different for a given substrate. The discrepant results of *in vitro* studies probably reflect the sensitivity of CYP3A metabolic activities to incubation conditions.

CYP3A7

CYP3A7 is the major CYP isoform detected in embryonic, fetal and newborn liver, but is also detected in adult liver, although at much lower levels than CYP3A4.[7,9,35] The CYP3A7 gene is also located on chromosome 7 and its nucleotide sequence is nearly 90% homologous to that of CYP3A4.[18,19]

Only a few substrates have been studied with regard to a role for CYP3A7 in their biotransformation. Formation of 1-hydroxymidazolam and carbamazepine-10,11-epoxide are only marginally supported by CYP3A7 as compared to CYP3A4.[5] The metabolism of zonisamide by CYP3A7 was approximately 70% of that by CYP3A4 activity.[34] In contrast, the biotransformation of cisapride to either norcisapride or its 2 primary ring-hydroxylated metabolites by CYP3A7 is at least 10-fold less than that observed with CYP3A4 under the same experimental conditions.[36]

CYP3A7 also plays an important role in the biotransformation of endogenous compounds. It catalyzes the 16α-hydroxylation of DHEA-S, a physiologically important reaction for the formation of estriol in pregnancy, with a higher affinity and maximal velocity than CYP3A4.[34,37] CYP3A7 is minimally involved in the 6β-hydroxylation of testosterone, although this conversion is essentially supported by CYP3A4.[5] Finally, CYP3A7 is capable of metabolizing potential environmental pollutants (e.g. aflatoxin B1).[5,38] Data derived from fetal liver microsomes may provide additional information on the role of CYP3A7 in the biotransformation of several substrates, since it is the main CYP isoform present in the fetal liver[35] (see table I).

Interindividual and intraindividual variation of CYP3A activity

Interindividual variation in CYP3A expression[3,21,39] is reflected by large interindividual differences (e.g. 4- to 13-fold) in plasma clearance of CYP3A substrates.[40-45] Using a human liver bank (24 adult Caucasian men and women), Transon et al.[29] found a 3.8-fold

(1.9 to 7.2 μmol/l) difference in the Michaelis-Menton constant (Km) and a 13-fold (9.5 to 123.4 nmol/mg protein/h) difference in the maximum rate of metabolism (V_{max}) for the 1'-hydroxylation of midazolam. Thummel et al.,[46] using a human liver bank assembled from organ donors (n = 21) found a 29-fold variation of V_{max} for midazolam 1-hydroxylation, whereas Kronbach et al.[47] only found a 5-fold variation. Thummel et al.[46] determined CYP3A content in intestinal microsomes from 15 donors and found an interindividual variation exceeding 18-fold for each region of the gut.

Intraindividual variation in midazolam plasma clearance [(CV%) as a measure of CYP3A activity *in vivo*] ranged from approximately 5 to 20%, which is considerably smaller than interindividual variation.[48] Ten adult patients, not receiving known CYP3A inducers, underwent two erythromycin breath tests within two weeks which showed a <27 % change in test results.[45] As reviewed by Fahr,[40] the pharmacokinetics cyclosporin exhibit up to a 2-fold intraindividual variability, which may partially be attributed to variability in CYP3A activity. In contrast, the interindividual variation in CYP3A7 mRNA in fetal liver was only 2.5-fold, which is considerably lower when compared with the 10-fold variation in CYP3A4 expression levels found in adult liver.[39]

Genetic variation in CYP3A expression

Racial and gender influences may explain part of the variability in CYP3A activity; however, study results remain inconclusive. In one study, no differences in hepatic CYP3A mRNA and protein were found between Caucasians and Japanese, but CYP3A-dependent activities appear to be higher in Caucasians when compared to Japanese.[3] Others could not detect differences in catalytic activity towards nifedipine, a well-known CYP3A substrate.[49] Recently, Chavez-Teyes et al.[50] have suggested that Mexican (Mestizo) males may have reduced CYP3A4 activity as compared with Caucasian males based upon higher midazolam bioavailability and reduced plasma clearance of midazolam in a small population (n = 11) of adults.

CYP3A activity measured *in vivo* by means of different probe drugs[51] was slightly higher in women when compared to men in two studies,[41,45] but other investigators could not confirm this gender difference.[48,52] Christians et al.[53] did not find a gender difference in the area under the concentration-time curve (AUC) after oral cyclosporin administration, but when coadministered with the CYP3A inhibitor diltiazem, the AUC of cyclosporine

was significantly more increased in women than in men. *In vitro* studies, using human liver microsomes, failed to support a gender difference in CYP3A activity,[3,39] but when using human intestinal microsomes, cyclosporine was metabolized significantly faster by microsomes from female than from male patients.[52] Finally, the menstrual cycle phase does not appear to influence CYP3A4 activity evaluated using midazolam in adults.[48] No correlation has been found between age or gender and heterogeneous CYP3A5 expression.[26,32]

To date, the large interindividual variation in CYP3A4 activity and expression could not be attributed to a genetic polymorphism of the CYP3A4 gene.[41] Recently, however, Rebbeck et al.[54] and Felix et al.[55] identified a mutation in the CYP3A4 promoter region which was associated with an increased severity of prostate cancer at diagnosis compared to patients without the mutation. The authors speculated that this may be because of CYP3A4 mediated differences in testosterone metabolism. They also reported a strong correlation between the incidence of secondary tumours after acute lymphocytic leukaemia in children and this CYP3A4 promoter region mutation. However the consequence of allelic variation in the CYP3A4 promoter region for CYP3A4 activity *in vivo* has not been addressed.
CYP3A5 cDNA sequencing revealed a point mutation, which was detected in 2 out of 5 individuals with absent CYP3A5 protein.[30] The authors suggest that one or more mutations may explain the heterogeneous expression of CYP3A5, but no additional studies have pursued this possibility.

Extrahepatic sites of CYP3A expression
The presence of CYP3A in other organs may also contribute to interindividual variability in the disposition of CYP3A4 substrates. CYP3A4 is expressed in esophagus, duodenum, small intestine and colon, but not the stomach, with the upper small intestine serving as the major drug metabolisingsite of the gastrointestinal tract.[15-17,56] In adults, approximately 40% of total CYP3A4 content is thought to reside in the small intestine.[57] Significant biotransformation of selected CYP3A4 substrates has been observed in the intestinal wall, considerably affecting the oral bioavailability of these drugs.[14,42,58-60] For example, when midazolam was given to healthy volunteers the extraction ratio for intestine and liver were comparable (approximately 40%) resulting in an overall oral availability of 30%.[46,60] Consequently, disease states that affect the intestinal epithelium

may reduce CYP3A4 activity, resulting in an increased bioavailability of CYP3A4 substrates.[61] In contrast, when midazolam was administered parenterally and delivered by arterial blood to the intestinal mucosa, only less than 10% of the delivered dose was extracted in the intestine, compared with almost 60% of the dose when delivered, after oral administration, to the luminal side of the intestine.[57]

Although intestinal CYP3A4 activity plays an important role in the first pass-extraction of many CYP3A4 substrates, interindividual variability in the activity of the intestinal transporter P-glycoprotein may be an equally significant determinant of oral bioavailability, at least for cyclosporin.[57] Moreover, overlapping substrate specificity exists for intestinal CYP3A and P- glycoprotein, which should also be accounted for when comparing disposition of CYP3A substrates after intravenous and oral administration.

CYP3A5 appears to be the main CYP3A isoform in the stomach and esophagus, although data are conflicting regarding the expression pattern of CYP3A5 in the remainder of the gastrointestinal tract. In general, depending on the analytical method used, CYP3A5 appears to be polymorphically expressed in the human intestinal tract, with considerably lower activities, when compared to CYP3A4.[15,16,56,62] CYP3A5 is the major CYP3A isoform detected in human kidney, lungs, blood and pituitary gland. Haehner et al.[63] detected CYP3A5 protein and mRNA in 100% of kidney samples. Interestingly, the investigators found a bimodal distribution in CYP3A5 content and activity. Renal CYP3A5 content and activity were 100- to 1000-fold lower than that found for hepatic CYP3A4.[63] Renal CYP3A4 mRNA was detected in 40% of kidney samples and in 70% of these samples, catalytically active CYP3A4 protein could be measured.[63]
CYP3A is expressed in neutrophils and B lymphocytes, but not T lymphocytes, although the analytical method was unable to distinguish between CYP3A4 and CYP3A5.[64] Since CYP3A content could not be induced by rifampicin (rifampin), it was suggested that the CYP3A isoform detected in human blood cells was more likely to be CYP3A5 than CYP3A4.[65,66]

Interestingly, CYP3A5 mRNA and protein were detected in the human pituitary gland and were located in growth hormone containing cells. This finding suggests that CYP3A may be involved in the regulation of growth hormone production.[67] Finally, low or negligible

CYP3A7 and CYP3A5 expression was found in extrahepatic embryonic and fetal tissue.[7,63,68]

CYP3A drug interactions

The vast majority of drug interactions mediated via CYP3A are the result of either induction or inhibition of this enzyme.[24] The effect of induction is to increase CYP3A4 content and to enhance drug clearance by this route.[69] CYP3A4 activity can be induced *in vitro* and *in vivo* by corticosteroids (e.g. dexamethasone), anticonvulsants (e.g. phenobarbital, phenytoin, carbamazepine) and several antimicrobials (e.g. rifampin, rifapentine).[70-76] The molecular mechanism of CYP3A induction has not been fully elucidated. Only recently, Lehmann et al.[77] identified a specific nuclear receptor in the CYP3A promoter region, which may explain the induction mechanism of CYP3A activity at least for some compounds. Inhibition of CYP3A4 activity *in vitro* and *in vivo* has been reported for several compounds such as imidazole derivatives, erythromycin, clarithromycin, troleandomycin, gestodene, ritonavir, fluvoxamine and grapefruit juice.[72,78-82]

Known inducers of CYP3A4, such as dexamethasone, rifampicin or phenobarbital, do not appear to induce CYP3A5 activity *in vitro*[26,27] Nevertheless, Schuetz et al.[83] showed that the CYP3A5 gene contains the sequence information (encoding a dexamethasone responsive element in the promoter region) needed for corticosteroid regulation of transcription. *In vitro* inhibition of CYP3A5 activity has been demonstrated for triazolam[34] and gestodene.[32] Ketoconazole and fluconazole are only weak inhibitors for CYP3A5 activity in human hepatocytes relative to their inhibiting effect on CYP3A4 activity.[84]

Pretreatment of HepG2 cells expressing human CYP3A7 with dexamethasone, rifampicin, troleandomycin, erytromycin, phenobarbital, phenobarbital-like inducers or lovastatin produced dose-dependent increases of CYP3A7 mRNA and immunoreactive protein.[24] Finally, rifampicin has been shown to induce CYP3A7 expression in adult human hepatocytes,[85] while gestodene inhibits its activity.[38]

6.4 Development of CYP3A activity

Although several exogenous factors are important determinants of interindividual variation in CYP3A activity, the impact of ontogeny on the activity of CYP3A isoforms contributes significantly from birth through adolescence. *In vitro* techniques have been used to assess the impact of development on CYP3A activity, but the majority of information currently available is derived from either pharmacokinetic studies of CYP3A substrates or the *in vivo* examination of biotransformation of CYP3A pharmacological 'probe' compounds.

Ontogeny of CYP3A activity assessed in vitro

The benefits and problems of the different methods used to determine CYP3A expression and activity *in vitro* have been discussed in recent reviews.[8,86] Briefly, problems with sensitivity or quantitation may arise, depending on the methods used to detect CYP expression. Specifically, studies which have determined total amounts of immunoquantifiable CYP3A protein from liver may not have used assays capable of differentiating the respective CYP3A isoforms.[49] Such limitations may explain conflicting results concerning the assessment of ontogeny of CYP3A activity *in vitro*. The limitations may now be overcome with the use of antibodies raised against isoform-specific peptide sequences or individual isoforms expressed in cells.[5]

Total CYP content

Total hepatic CYP content appears to be fairly stable from early fetal life to the first year after birth with reported levels between 30% and 60% of adult levels.[87-89] Other authors, [9,21] however, reported levels in neonates and infants similar to those found in adults.

CYP3A content

Fetal CYP3A content (immunoquantitated from liver microsomes) ranges from 30% to 100% of adult CYP3A content.[89,90] An early report showed that total CYP3A content represents 85% of total CYP content in fetal liver, increasing in parallel with the total amount of CYP during development.[87] However, another study specifically measuring CYP3A7 showed that its content represents only around 30% of total fetal CYP content.[89] During the transition from fetal to neonatal life total CYP3A content appears to be relatively stable.[5]

CYP3A4

In embryonic hepatic tissue (6-12 weeks gestational age) CYP3A4 mRNA could not be detected.[39] In contrast, CYP3A4 mRNA was detected in fetal liver microsomes (at between 11 and 30 weeks gestational age) at 10% of adult levels, increasing immediately after birth and reaching approximately 50% of adult levels between 6 and 12 months of age.[5,85,91] As was shown by Lacroix et al.[5] testosterone β-hydroxylase activity in human liver microsomes, mainly an activity of CYP3A4, as extremely low in the fetus with an increase after birth reaching 30-40% of adult levels from 3 months to one year of age and 120% of adult levels after the age of 1 year. Gestational age at birth (25 to 40 weeks) did not have an influence on this ontogenic pattern of CYP3A4 activity.[5]

CYP3A5

CYP3A5 is consistently demonstrated in embryonic liver.[39] However, CYP3A5 protein could be detected in only 10% of fetal livers.[32] Gender does not appear to affect fetal expression of CYP3A5.[39] Wrighton et al.[32] showed that CYP3A5 was expressed in nearly 50% of all infant livers studied, while in the same study expression of CYP3A5 was only found in 29% of adult livers. CYP3A5 protein was detected in the livers of a 2-month-old and a 14-year-old patient in another study.[27] Given the dependence of CYP3A5 quantification on various (i.e. different) analytical methods, the aforementioned results provide a useful, albeit limited, picture of CYP3A5 ontogeny.

CYP3A7

CYP3A7 constitutes about 32% of total CYP content in the human fetal liver[89] and is not detected in other organs during embryogenesis (days 50 to 60).[7] The hydroxylation of DHEA-S, which is mainly catalyzed by CYP3A7 and to a much lesser extent by CYP3A4, was considerably higher in liver samples from 10- to 20-week fetuses, when compared with a liver sample from a term infant.[5,37] Immediately after birth, DHEA-S hydroxylation more than doubled, with the highest activity being reached between postnatal day 1 and 7. The activity decreased dramatically after the first week of life to only 10% of newborn levels between 3 and 12 months of age.[5] Reported CYP3A7 mRNA expression in human adult livers ranges from 54% to almost 90% of samples.[39,91] However, the amount of CYP3A7 mRNA detected was only 1.7% to 10% in adults when compared to the fetus.[39,85]

CYP3A7 mRNA and protein are also detected in adult endometrium and placenta and in higher amounts in the endometrium of pregnant women. The placental and endometrial CYP3A7 content appears to increase significantly from the first to the second trimester of pregnancy.[92] CYP3A7 content in placenta and endometrium per gram of tissue is between 0.6 and 5.5% of CYP3A7 content in fetal liver. During gestation, the contribution of CYP3A7 in endometrium and placenta to the metabolism of substrates for this isoform should not be underestimated since placenta weight is approximately 5 times more than total fetal liver weight .

In summary, CYP3A7 activity is high before birth, whereas CYP3A4 activity is very low. Directly after birth a transition from predominantly CYP3A7 activity to mainly CYP3A4 activity occurs Fig. 1.[5]

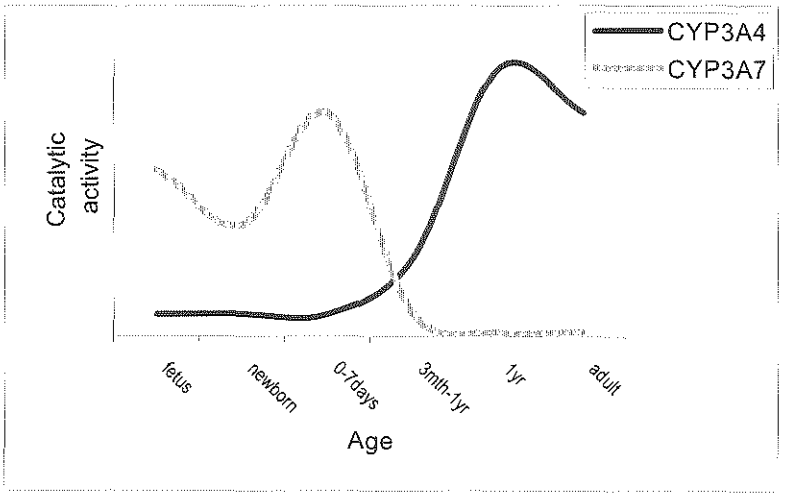

Figure 1 *Ontogeny of cytochrome P450 (CYP)3A4 and 3A7 activity expressed as activity measured using isoform-specific probes in human liver microsomes.*

Finally, Table II summarises *in vitro* studies on CYP3A-mediated metabolism during development.

Table 2 Substrate specificity of the 3 cytochrome P450 (CYP) 3A isoforms and CYP3A-mediated metabolism during development *in vitro*[24,25]

	Isoforms expressed in cells			Catalytic activity towards CYP3A substrates in human liver microsomes[a]					
Drugs	**3A4**	**3A5**	**3A7**	**adult (%)**	**child (%)**	**newborn (%)**	**fetus (%)**	**references**	**comments**
Carbamazepine	+++	++	+	Present			CBZ-E formation (?)	34, 93	Age-dependent change in metabolites formed CBZ-E present in stillborn fetus of mother receiving carbamazepine
Cyclosporin (M1 formation)	+++	+++						28	
Cyclosporin (M17 formation)	+++	ND						28	3A5, less metabolites formed
Dextromethorphan				100			30	94,95	3 MM formation (CYP3A)
Diazepam (minor)				100	140 (3-12 mo)	15 (<24h postpartum) 40-50 (1-7days postpartum)	<5	96	Temazepam formation rate (CYP3A mediated)
Erythromycin	+++	ND/+++						32,33	
Ethylmorphine	+	++		100			100	33,68	
Indinavir				100	67 (6-11y)		32	91	
Lidocaine	++	+++	?					97	
Midazolam (1-hydroxy formation)	+	+++	+/-					5,31	
Midazolam (4-hydroxy formation)	++	+++						31,98	
Nifedipine	+++		+++	100	44		18	28,32,90	
Paracetamol (acetaminophen)				100			10	99	Also CYP2E1, sulphation and glucuronidation
Quinidine	+++		ND					32	
Zonisamide	+++	+	++					34	
Endogenous substrates									
Androstanedione (6β-hydroxylation)	+++	++	++					28,100	
Cortisol	+++	++	-					32,101	
DHEA (16α-hydroxylation)	+		+++	+			+++	5,32,37,87	
DHEA (16β-hydroxylation)				+++			+	87	
DHEA-S	++	++	+++					32,34	
Progesterone (6β-hydroxylation)	+++	++						28,39	
Testosterone (2β-hydroxylation)	+++	++	++ / ++++	<1% or 100	12-38		12	5,27,34,90	
Testosterone (6β-hydroxylation)	+++	++	+	100	30-40%	30-40	2-10	89,100,102	
Testosterone (15β-hydroxylation)	+++	+/-						28	
Xenobiotics									
Aflatoxin B1	+++		ND	100			200	23,38,89,103	activation of aflatoxin B1 *in vitro*
Benzopyrene activation	Activity?		+++	100			600	89	
Benzphetamine	+++		+++					33,35	
Heterocyclic amines	+	++	+++					104	Activation of IQ and MeIQ, from cooked meat, fish and tabacco
Sterigmatocystin	+++		+++	100			50	38,89	

a Relative to adult rate = 100 % ***CBZ-E*** *= carbamazepine-10,11-epoxide;* ***DHEA*** *= dehydroxoepiandrosterone;* ***DHEA-S*** *= dehydroxoepiandrosterone 3-sulphate;* ***IQ*** *= heterocyclic amine: 2-amino-3-dimethyl-imidazo[4.5-f]quinidine; MeIQ = heterocyclic amine; 2-amino-3.4-dimethyl-imidazo[4.5-f]quinidine;* ***3MM*** *= 3-methoxy-methorphan;* ***ND*** *= not detected; + to ++++ indicate increasing levels of expression; - indicates note expressed;* ***?*** *indicates expression unknown.*

Ontogeny of CYP3A activity in vivo

As reviewed by Watkins,[51] CYP3A activity can be assessed *in vivo* with the use of different pharmacologic probes. According to the validation criteria for non-invasive probes for CYP3A4 activity as defined by Watkins,[51] the erythromycin breath test (ERMBT) is currently one of the best validated methods to assess CYP3A activity *in vivo*.[105] The ERMBT results correlate significantly with the plasma clearance of orally given cyclosporin[106] and intravenously administered midazolam,[105] the latter also correlates with *in vitro* CYP3A4 content and catalytic activity.[46,60] Furthermore, the plasma clearance of midazolam significantly correlates with that of nifedipine[107] and nifedipine oxidation activity *in vitro* is significantly correlated with CYP3A4 protein levels.[49]

Another important CYP3A probe is the urinary 6β-hydroxycortisol : cortisol (6βOHF :C) ratio which, however, does not correlate with either ERBMT or midazolam clearance.[108,109] As discussed by Watkins[51] a possible explanation for this lack of correlation may be that renal CYP3A5 also catalyzes the metabolism of cortisol and therefore, urine-based assays such as the 6βOHF:C ratio may not correctly reflect hepatic CYP3A activity. Nevertheless, cortisol is frequently used as a probe to assess CYP3A induction.[72]

Because of the intravenous use of [N-methyl^{14}-C]-erythromycin, the ERBMT has formidable ethical obstacles to overcome before it can be used in children. An attractive alternative is the use of oral ^{13}C-labeled (stable isotope) erythromycin which, because of technical difficulties has not yet been used to study CYP3A activity in this population. In contrast, the plasma clearance of erythromycin does not provide information on CYP3A activity since the drug is mainly cleared unchanged into bile and only a small part proceeds by hepatic *N*-demethylation.[110]

The plasma clearance of midazolam has also been used to assess CYP3A activity. Jacqz-Aigrain et al.[111-113] studied the pharmacokinetics of midazolam in premature and term neonates. The plasma clearance of midazolam was significantly reduced in newborn infants (0.11 to 0.13 l/h/kg) when compared with that of adults (0.38 to 0.66 l/h/kg)[112,114] and was even lower in preterm infants younger than 39 weeks gestational age (0.072 to 0.096 l/h/kg).[5,112,115] Since midazolam is only slightly metabolized by CYP3A7,[31] the

reduced clearance of midazolam in the newborn may be explained by developmentally low CYP3A4 activity following birth.[5] A marked increase in midazolam clearance (per kg bodyweight) in the first weeks of life, as may be expected to occur in parallel with a developmentally regulated surge in CYP3A4 activity, is not, however, observed. Conflicting data exist on midazolam clearance after the newborn period. In one study, clearance was similar in older infants, children (0.48 l/h/kg)[116] and adults (0.38 to 0.67 l/h/kg)[114,117] while in another, Hughes et al.[118] found lower clearances in children (0.14 to0.18 l/h/kg) until 2 years of age. In older children, however, (3 to 13 years old) midazolam clearances were higher (0.78 l/h/kg) than those observed in adults (0.38 to 0.66 l/h/kg). These changes are summarised in figure 2.

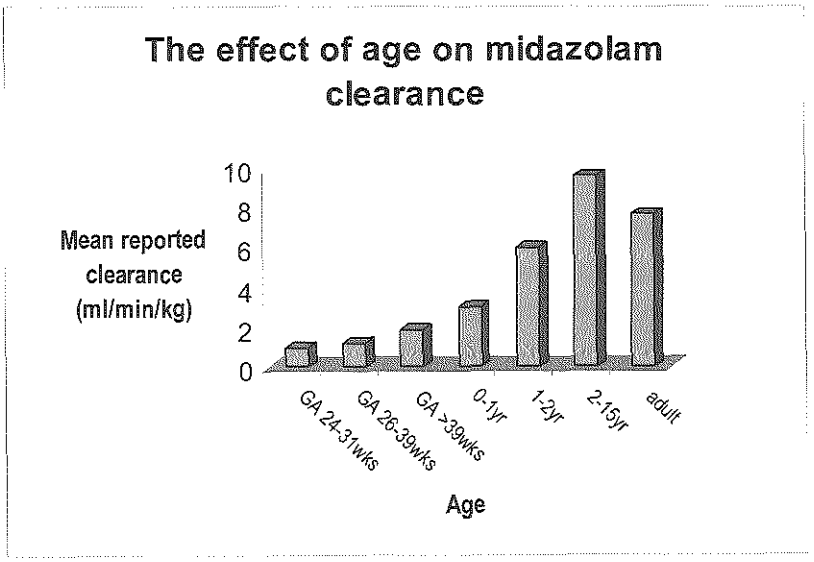

Figure 2 *Effect of age on the mean clearance (corrected for body size using bodyweight) of midazolam after intravenous administration*[112,114,116-123]
GA = gestational age, postnatal age <15 days

The oral clearance of midazolam has been proposed as a measure of combined hepatic and intestinal CYP3A activity in adults.[51,57] Although the cDNA of hepatic and intestinal CYP3A4 is similar,[58] they appear not to be regulated in concert.[124] Therefore, it is unclear if intestinal CYP3A activity changes in parallel with hepatic CYP3A activity during ontogeny. Oral midazolam bioavailability is approximately 36% in adults,[125] somewhat higher than that found in children where bioavailability ranged from 15% to 27% with

doses ranging from 0.15 to 1.0 mg/kg.[126] No data are currently available on the oral clearance of midazolam during the first weeks of life.

Cortisol is hydroxylated by CYP3A4 and CYP3A5[127,128] and to a much lesser extent by CYP3A7.[101] The 6βOHF : C ratio has been used to study CYP3A activity in neonates up to 1 year of age. Nakamura et al.[129] measured the 6βOHF : C ratio at different time points during the first 2 weeks of life. Within 24 hours of after delivery, the ratio was significantly larger compared to adults and declined to adult levels within 3 days after birth. A similar pattern of urinary 6βOHF : C ratios was found by Vauzelle-Kervoedan et al.[130] in term infants at a single occasion between 1 and 15 days after birth. In infants from 1 to 12 months of age, the mean 6βOHF : C ratio was lower compared with that of neonates and adults. No correlation could be found between the high 6βOHF : C ratio of term neonates on the day of birth and the 6βOHF : C ratio of their mothers, measured 2 hours after delivery.[128] The authors speculate that, given their observation that cortisol is also metabolised by CYP3A7 and that CYP3A7 activity is high directly after birth,[5] the observed elevated 6βOHF : C ratio seen on the first day of life may be caused by age-related changes in CYP3A7 activity. This is, however, in marked contrast to the low testosterone 6β-hydroxylase activity observed *in vitro* in fetal livers, CYP3A7 has a higher catalytic activity towards testosterone than cortisol *in vitro*.[5]

The 6βOHF : C ratio in premature infants was significantly lower directly after birth, than in term infants, with no change during the first 2 weeks of life.[129] It is unclear if these differences in 6βOHF: C ratio between preterm and term infants accurately reflect the differences in CYP3A4 activity during the first two weeks of life. Lacroix et al.[5] did not find a gestational age related (25 to 40 weeks) difference in postnatal development of CYP3A7 and 3A4 activity in human liver microsomes. Since the reduced renal function seen in preterm infants may reduce the renal clearance of cortisol and its metabolite, gestational age and postnatal age-related changes in CYP3A activity may be obscured.

Nifedipine is a well-defined CYP3A substrate and its plasma clearance may therefore be associated with CYP3A activity.[51] Pharmacokinetics of oral nifedipine were studied in children with bronchopulmonary disease aged 5 to 68 months. The elimination half-life ($t_{½β}$) of nifedipine was lower in children (1.8 h.) when compared with the previous data in adults (2.4 to 3.4 h).[131] As pointed out by Kearns,[25] the plasma clearances values varied

considerably, which obscured age-dependent changes (from 5 to 68 months of age) in clearance.

Lidocaine is also metabolized by CYP3A4[97] to its main metabolite monoethyl-glycinexylodide (MEGX). The MEGX formation rate has been proposed as a general test for hepatic dysfunction (as was recently reviewed by Tanaka[132]). However, the use of MEGX formation rate as a measure of CYP3A4 activity *in vivo* seems less appropriate, since only few of the criteria for a CYP3A probe, as discussed by Watkins et al.[45] have been met. Moreover, lidocaine is a high extraction drug, and variability in its clearance may therefore be more appropriately explained by hepatic blood flow differences than by variation in CYP3A activity.[51]

Pharmacokinetics of important CYP3A substrates in children

Cyclosporin

Cyclosporin is a widely used immunomodifier in paediatric transplant recipients. In their comprehensive review, Cooney et al.[133] discussed the age-related differences in cyclosporin pharmacokinetics in transplant recipients. Although the apparent volume of distribution does not appear to change as a function of age, cyclosporin plasma clearance (on a per kg or body surface area basis) is considerably higher in paediatric patients younger than 8 years when compared to adults. As a result, age-specific cyclosporin dosage requirements are necessary to prevent allograft rejection.[134,135]

This increased dose requirement for cyclosporin in children is suggestive of increased hepatic CYP3A activity in this age-group. Furthermore, as cyclosporin is also metabolized by intestinal CYP3A,[136] developmental changes in intestinal CYP3A activity may also contribute to the observed lower oral bioavailability in paediatric patients as opposed to in adults.[133] Differences in surgical approach to liver transplantation between children and adults may also contribute to higher dose requirements in children.[133] Moreover, it is possible that age-dependent expression and activity of P-glycoprotein, which is also located in the intestinal wall, may contribute significantly to the presystemic clearance of cyclosporin.[137] As illustrated by a recent case report where CYP3A activity was induced following rifampin administration,[76] the altered activity of CYP3A, which occurs consequent to development, may be of significant magnitude to impact upon the toxicity profile of this drug.

Tacrolimus

Tacrolimus (FK 506) is also an immunosuppressant used in paediatric patients to prevent post-transplant allograft rejection. As with cyclosporin, a correlation appears to exist between whole blood concentration of tacrolimus and both efficacy (i.e. no rejection) and toxicity in adults and children.[138] Yasuhara et al.[139] found marked interindividual variability in the pharmacokinetics of tacrolimus in children aged between 3 months to 15 years after liver transplantations. Part of this variability was explained by a decrease in clearance (per kg bodyweight) of tacrolimus with increasing bodyweight and an increase in clearance with postoperative days. This finding supports the observation that children younger than 3 years old need 2 to 3 times higher oral doses of tacrolimus per kg bodyweight to maintain the same plasma trough concentrations as older children and adults.[139]

Filler et al.[140] who studied the disposition of tacrolimus in renal transplant patients, also found considerable interindividual variation in AUC values after oral administration of tacrolimus. Therefore, they recommend therapeutic drug monitoring of tacrolimus in paediatric patients. Interestingly, they did not find substantial pharmacokinetic differences in tacrolimus disposition in this group of patients when compared with adult data, which may be explained by the older age of the children studied and the inclusion of young adults (age range was 5 to 23 years).

Etoposide

After oral administration of the antineoplastic agent etoposide (VP-16) in paediatric patients between 1.1 and 17 years of age, the AUC and halflife values were only slighter lower than those reported for adults.[141] However, the systemic clearance of etoposide was not significantly altered by the age in contrast with disease-related changes in serum albumin, which accounted for 46% of the variability of etoposide clearance.[141] Likewise, Boos et al.[142] did not observe any age-related changes in the clearance of etoposide, in children and young adults between 3 months and 28 years of age when corrected for bodyweight or body surface area.[142]

Carbamazepine

CYP3A4, and to a lesser extent CYP2C8, catalyze the biotransformation of carbamazepine to its main metabolite carbamazepine-10,11-epoxide.[74] The observation of

this metabolite at very low concentrations when compared to carbamazepine concentrations in the circulation of stillborn fetuses of mothers receiving carbamazepine may be interpreted as evidence that carbamazepine is metabolised by CYP3A7.[143] A significant correlation was found between age and carbamazepine dose ratio (reciprocal of apparent oral clearance) in patients with epilepsy, ranging between 3 months and 29 years of age.[144] In addition, Korinthenberg et al.[145] found an inverse relationship between the ratio of the epoxide metabolite to carbamazepine and age in children ranging in age from 2 weeks to 15 years who were at steady state, either on carbamazepine monotherapy or with concomitant administration of other aromatic anticonvulsants capable of inducing CYP.

Astemizole

Shortly after its introduction, the histamine H_1- receptor antagonist astemizole, a CYP3A4 substrate,[24] was associated with cardiotoxic effects in children following accidental overdosing of this drug. Several investigators reported a prolonged QT interval, ventricular dysrrhythmia, atroventricular block and brief episodes of ventricular tachycardia and fibrillation.[146-148] However, no data are available on the pharmacokinetics of astemizole in children, so the effect of coadministration of a CYP3A inhibitor or predisposition for a prolonged QT interval cannot be determined at this moment.

Cisapride

The prokinetic drug cisapride is widely used in young children for the treatment of gastro-oesofageal reflux. The biotransformation of cisapride is mediated by CYP3A4 with little contribution of the other CYP3A isoforms.[36] Reports of prolonged QTc interval and in some cases serious ventricular arrhythmias, when cisapride was ingested with known CYP3A4 inhibitors[149-151] suggest that reduced CYP3A4 activity present in young infants[5] may predispose them to concentration-related adverse effects. Despite reduced CYP3A4 activity in the first month of life, an increased clearance of cisapride (normalized to bodyweight) was seen in premature infants with postnatal ages from 15 to 199 days when compared to the dose requirements with adults. There was no evidence of significant increases in the plasma cisapride concentrations.[151] However, further clinical studies will be needed to clarify the association between the pharmacokinetics of cisapride and the consequences of a prolonged QT interval in preterm infants.

Caffeine

In the neonate, CYP1A2 activity is severely reduced, while CYP3A isoforms are present. Cazeneuve et al.[152] demonstrated that the biotransformation of caffeine in the neonate is primarily dependent on CYP3A and not on CYP1A2, as is the case in older infants and adults. Moreover, in contrast with the adult, 85% of a caffeine dose is excreted unchanged by the kidney in neonates.[153] Consequently, reduction in the activity of CYP1A2 and CYP3A activity early in life is less important than renal function with respect to caffeine pharmacokinetics in the neonate.[152-154]

6.5 Inhibition and induction of CYP3A activity during development

In vitro inhibition studies with known CYP3A substrates (midazolam, DHEA and progesterone) have revealed significant inhibition of ethylmorphine metabolism.[68] Significant inhibition of CYP3A7 expressed in COS cells was seen for triazolam, but not for troleandomycin.[34] Tateishi et al.[9] detected CYP3A7 protein in 2 out of 10 livers of patients aged 2 years and older (8 and 10 year of age, respectively). The authors speculated that the long term treatment of these children with drugs known to induce CYP3A activity may have induced their CYP3A7 expression. However, CYP3A7 mRNA but not functional protein was induced by rifampicin in human adult hepatocytes.

No controlled trials have studied the effect of inhibitors or inducers on CYP3A activity in children. Some limited information can, however, be gained from examination of relevant case reports. Sereni et al.[155] reported that in neonates who had received phenobarbital during fetal life or early after birth, the urinary excretion of diazepam metabolites was significantly higher than in neonates who had not. Treluyer et al.[96] showed that in fetal liver microsomes from the offspring of mothers who received a CYP3A inducer (e.g. phenobarbital or prednisone), concomitedly with diazepam, the formation of both temazepam (CYP3A-catalyzed) and *N*-desmethyldiazepam (CYP2C-catalyzed) were dramatically increased when compared with controls.

Hiller et al.[156] described the case of an 8-year old boy who received an oral doze of midazolam 0.5mg/kg as premedication, followed 1 hour later by an erythromycin infusion. Shortly thereafter he lost consciousness which was subsequently regained after

discontinuation of the erythromycin, a known CYP3A4 inhibitor. As expected, the plasma concentrations of midazolam were significantly elevated when erythromycin was coadministered. Hughes et al.[118] reported a similar inhibitory effect of erythromycin on the kinetics of midazolam, resulting in reduced consciousness in a child.

Cyclosporin produced a 2-fold increase in the AUC of the CYP3A substrate etoposide when given as combination chemotherapy for solid tumors in children[157,158] The inhibitory effect of cyclosporin on clearance of etoposide is similar in children when compared with adults[24] and suggestive of competitive inhibition of hepatic CYP3A by cyclosporin. The authors advised reducing etoposide dose by 50% when given to paediatric patients in combination with cyclosporin.[158]

The $t_{½\beta}$ of carbamazepine in neonates exposed to the drug *in utero* was comparable with that seen in adults.[159] Given the fact that CYP3A4 activity is considerably lower in neonates[5] and that all mothers in this study also received phenytoin, it would appear that CYP3A isoforms in the fetus and neonate are inducible.[159] Detection of carbamazepine-10,11-epoxide in fetuses of mothers who received carbamazepine before delivery not only suggest a role for CYP3A7 in the epoxidation of carbamazepine, but also the inducibility of this process. However, it should be noted that carbamazepine-10,11-epoxide concentrations are also determined by the activity of the drug-metabolising enzyme epoxide hydrolase which further metabolises the epoxide, and that the activity of this enzyme may also be altered by drugs such as phenytoin.[160]

Finally, carbamazepine concentration : dose ratios were decreased, and formation of the 10,11-epoxide metabolite was significantly increased in paediatric patients who received phenytoin in addition to phenobarbital.[161] Despite clear evidence which demonstrates that CYP3A isoforms are inducible during development, the impact of ontogeny on the rate and/or extent of such interactions is not generally known.

6. 6 Regulation of CYP3A activity during ontogeny

Little is known about the factors that govern CYP3A7 expression in the fetus and the transition from CYP3A7 to CYP3A4 activity after birth. Hashimoto et al.[22] showed that

both CYP3A7 and 3A4 have characteristic sequences in the 5'-flanking region with an identity of 91%. The 5'-flanking region of CYP3A5 is only 74% related to both CYP3A4 and 3A7.[162] Several consensus sequences for transcription factors (e.g. estradiol response element, CAAT box) were found in all three 5'-flanking regions. Moreover, the 5-flanking region of CYP3A has specific deletions and/or sequences which seem characteristic for each gene. However, whether these sequences and/or deletions are involved in the regulation and differential expression of the individual CYP3A genes during development remains to be established.

Given the ability of human growth hormone to modulate the effect of many general transcription factors, and the effect of human growth hormone to enhance CYP3A-mediated drug metabolism, growth hormone may be an important factor in the transcription of CYP3A, as discussed by Leeder et Kearns.[10] Liddle et al.[163] showed recently that human growth hormone and also dexamethasone regulate CYP3A4 gene expression, possibly at the pretranslational level, in cultured human hepatocytes. Moreover, they demonstrated an inhibitory effect of tri-iodothyronine on the transcription of the CYP3A4 gene. The role of these hormonal factors in the regulation of CYP3A4 expression during development needs, however, to established.

6.7 Ontogeny of CYP3A activity and the impact on drug development

The CYP3A family is involved in the biotransformation of numerous therapeutically important drugs. Clearly, distinct ontogenic patterns exist for the 3 isoforms of this subfamily. Because of the difficulty in obtaining liver and other tissue material from children, scarce data are available on the ontogeny of the individual CYP3A isoforms in the neonatal period, infancy and childhood. Moreover, data on the disposition of individual substrates and their specific metabolic profile at different phases of ontogeny are lacking for many CYP3A substrates. Accordingly, application of existing data to modify the design of pharmacokinetic studies of CYP3A substrates, inducers or inhibitors is limited in most instances to inferential information gleaned from individual published studies. Given the apparent lack of correlation between liver size and hepatic microsomal enzyme activity,[164] and between hepatic CYP activity and the amount of hepatic microsomal protein,[165] caution must be exercised when plasma drug clearance is used to

assess the impact of development on CYP3A activity. More specifically, when extrapolating pharmacokinetic data for CYP3A substrates in relation to CYP3A activity, it is important to note that corrections made for bodyweight or body surface area may not accurately reflect age-related differences in drug metabolism.[166]

In vitro[5] and *in vivo*[25] data clearly support a marked reduction in the activity of CYP3A4/5 during the first 1 to 2 months of postnatal life. However, during infancy, and for the first 2 to 3 years of life, CYP3A4 activity appears to exceed adult values as reflected by clearance of midazolam,[118] cyclosporin[133] and tacrolimus.[139] When these data are converted using the allometric model,[2] the age-dependent changes in clearance persist, which is suggestive of elevated CYP3A activity in this age group. Accordingly, pharmacokinetic data would appear to support at least 2 developmental 'breakpoints' for CYP3A activity during the first 3 years of life. It must be noted, however, that these apparent developmental 'patterns' of CYP3A activity may be significantly influenced by both exogenous (e.g. drugs capable of altering CYP3A expression, activity and /or the access of a substrate to the enzyme) and endogenous factors (e.g. intrinsic hepatic disease or the effect of cytokines associated with systemic infection on CYP3A activity[132,167]). Such factors, depending upon the direction and magnitude of effect, could well obscure the apparent impact of ontogeny.[10] Nonetheless, the design of pharmacokinetic studies and ultimately of dosage regimens for CYP3A substrates during the first 2 to 3 years of life must capture the developmental pattern of enzyme function so as to ensure adequate characterisation of the disposition characteristics of a drug and the translation of this information into the provision of safe drug therapy.

6.8 Future directions

Clearly, several critical information gaps exist with regard to the overall impact of ontogeny on CYP3A activity. First, the pharmacokinetically important CYP3A substrates should be completely characterized over the first 3 to 5 years of life. Also, other important drugs used in the neonatal period should be studied in order to clarify a possible 'compensatory' role for CYP3A when the CYP isoforms may be markedly reduced. Secondly, the factors governing the transition from CYP3A7 to 3A4 around birth should be elucidated, as this may provide insight into the genetic and possibly, neurohumoral

factors, that upregulate the activity of this and possibly other CYPs.
Thirdly, the role of both hepatic and renal CYP3A5 on xenobiotic disposition must be better characterised. Several authors suggest that the lack of correlation between different probe drugs to assess CYP3A activity may be because of the differential contribution of hepatic and renal CYP3A5 to their metabolism. This is a potentially important consideration when CYP3A4 activity is low.

Finally, the role of intestinal CYP3A and the impact of ontogeny on its activity must be studied because of its potential importance in determining presystemic drug clearance. It is quite possible that age-dependent differences in the rate and/or extent of oral bioavailability for CYP3A substrates may be more a function of changes in the activity of intestinal CYP3A than age-associated differences in gastrointestinal motility.

6.9 Conclusion

Profound developmental differences in the activity of CYP3A isoforms occur *in utero*, in the neonatal period, through infancy and early childhood and to adolescence. In many instances these changes have proven to be of clinical significance with respect to treatment using substrates, inhibitors or inducers of CYP3A. Investigators and clinicians must consider the impact of ontogeny on CYP3A in both the design of studies and interpretation of pharmacokinetic data, as well as in the provision of therapy to paediatric patients.

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Chapter 7

The Single-Dose Pharmacokinetics of Midazolam and its Primary Metabolite in Pediatric Patients after Oral and Intravenous Administration

Based on the article:

The Single-Dose Pharmacokinetics of Midazolam and its Primary Metabolite in Pediatric Patients after Oral and Intravenous Administration

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7.1 Abstract

The first-dose pharmacokinetics of midazolam and its primary α-hydroxy metabolite were studied after a single-dose administration. Eligible study patients were enrolled into one of three study arms: Arm I (midazolam/metabolite pharmacokinetic evaluation after oral administration of a syrup formulation), Arm II (the absolute bioavailability of midazolam syrup) and Arm III (midazolam and metabolite pharmacokinetics after IV administration). Complete blood sampling for pharmacokinetic analysis was available in 87 subjects. Midazolam absorption after administration of the oral syrup formulation was rapid, with adolescents absorbing the drug at approximately half the rate observed in younger children (ages 2 to < 12 years). Furthermore, midazolam $t_{1/2}$ was prolonged and Cl/F reduced in adolescents as compared with younger children. Although the midazolam V_d/F appeared larger in the youngest age group after oral administration, this observation was not apparent after IV dosing, suggesting subject differences in bioavailability rather than distribution. Like midazolam, the disposition characteristics for α-hydroxymidazolam were also highly variable, with the greatest formation of metabolite (reflected by AUC ratio) observed in children ages 2 to < 12 years. The AUC ratios of α-hydroxymidazolam to midazolam after IV dosing were similar across all age groups and were smaller than corresponding values following oral administration. The absolute bioavailability of midazolam averaged 36 % with a very broad range (9 % - 71 %). No relationship between midazolam bioavailability and ages was observed. Overall, the disposition characteristics of midazolam and its α-hydroxy metabolite were highly variable, appeared independent of age and dose administered, and were linear over the dose range studied (0.25 to 1 mg/kg). These data suggest that an initial oral dose of 0.2 to 0.3 mg/kg should be adequate for successful sedation of most pediatric patients. The inherent variability in midazolam bioavailability and metabolism underscores the importance of titrating midazolam dose to desired effect.

7.2 Introduction

Benzodiazepines have been prescribed for more than three decades to patients of all ages for a wide array of physical and psychosocial disorders. These agents are safe and well tolerated across a broad dose range. Depending on the dose administered,

benzodiazepines produce sedation, hypnosis, anxiolysis, muscle relaxation, antegrade amnesia, and possess anticonvulsant activity.[1-3] Nearly all of the clinical effects of benzodiazepines result from their actions on the GABA receptor within the central nervous system (CNS). The GABA receptor is an integral membrane-bound chloride channel responsible for most of the rapid, inhibitory neurotransmission in the CNS.[4] The effective and predictable sedative, muscle-relaxant, and antegrade amnesia effects of benzodiazepines support their widespread clinical use in pediatrics.[3,5]

Midazolam is one of the most extensively used benzodiazepines in pediatric practice.[3,6-15] Compared with other analogs of this class, the drug possesses a rapid onset and short duration of action. Moreover, midazolam is a water-soluble compound, which eliminates the need for propylene glycol in the manufacture of the parenteral formulation. Propylene glycol, a component of other benzodiazepine formulations (e.g., diazepam, lorazepam), is associated with adverse events such as phlebitis.[3] Midazolam is currently available in the United States as an injectable solution for intravenous (IV) and intramuscular (IM) administration in adult and pediatric patients. An oral syrup formulation of midazolam was recently approved for use in pediatrics.

The pharmacokinetics of midazolam are well characterized in adults and children after parenteral administration.[1,16-19] Pharmacokinetic information following oral administration in pediatric subjects is limited primarily to data obtained using oral solutions prepared extemporaneously from the parenteral formulation.[3,20] Prior to the availability of an oral syrup formulation of midazolam, physicians routinely administered midazolam orally to pediatric patients using the parenteral formulation mixed with a sweetener or juice.[3,20-22] The purpose of the present investigation was to determine the pharmacokinetics of midazolam and its primary metabolite, α-hydroxymidazolam, after IV administration and after oral administration of the new syrup formulation across a broad dose range. The pharmacokinetic evaluation presented in this report was part of a large randomized, prospective pharmacodynamic-pharmacokinetic study of oral midazolam syrup in pediatric patients.[23]

7.3 Methods

Patient Inclusion and Exclusion Criteria

Pediatric patients 6 months to < 16 years of age who were scheduled to receive sedative premedication prior to minor in-hospital or day-stay procedures were eligible for enrollment into this study. Study patients must have been of ASA physical status I, II or, III and between the 10^{th} and 95^{th} percentile for weight and height. Study patients were excluded from enrollment if they were pregnant or nursing; required any concurrent sedative medication less than 8 hours before midazolam dosing; had a known hypersensitivity to benzodiazepines, cherry, or cherry products (the primary flavoring ingredient of oral midazolam syrup); had any evidence of upper airway disease, CNS dysfunction, gastroesophageal dysmotility, or reflux disease; or were receiving acute or chronic medication(s) that could interfere with midazolam disposition (e.g., hepatic cytochrome P450 2D6 and/or 3A3/4 inducers, inhibitors or substrates).[23] Eight centers participated in patient enrollment; (Case Western Reserve University, Cleveland, OH; University of Missouri-Kansas City, Kansas City, MO; University of Tennessee, Memphis; Louisiana State University, Shreveport; University of Arkansas Health Sciences Center, Little Rock, AR; Ohio State University, Columbus, OH; University of California-San Diego, San Diego CA; University of Texas, Houston, TX). The first seven of these study sites are members of the National Institute of Child Health and Human Development Pediatric Pharmacology Research Unit (PPRU) network. The conduct of this study was approved by the institutional review boards for human subject investigation of each participating institution. Signed, written informed consent was obtained from the parent(s)/legal guardian(s) of each patient and whenever possible, assent was obtained from children ≥ 8 years of age before participation in the study.

Within 7 days of enrollment and before midazolam administration, each study patient provided a complete medical history and underwent a complete physical examination. A blood sample was obtained for the determination of serum creatinine (SCr), urea nitrogen, glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, total and direct bilirubin, and a complete blood count. A serum or urinary pregnancy test was performed for all menstruating female patients. Laboratory evaluations were performed by the clinical laboratories of the participating institutions; laboratory tests were repeated only if clinically indicated by the investigator or patient's physician.

Drug Administration and Sample Collection

Eligible study patients were enrolled into one of three study arms for drug dosing. In Arm I, the pharmacokinetics of midazolam and α-hydroxymidazolam were assessed after oral administration of midazolam syrup. Patients were stratified by age group (i.e., 6 months to < 2 years, 2 to < 12 years, and 12 to < 16 years) and randomly assigned to receive a single dose of midazolam, 0.25, 0.5, or 1 mg per kg of body weight (up to a maximum dose of 40 mg). Investigators and patients were blinded to the dose strength administered. In Arm II, the absolute bioavailability of midazolam syrup was assessed using an open-label, two-way crossover design with patients stratified into the same age groups as Arm I. Patients received a single IV dose of midazolam (0.15 mg/kg) and a single oral dose of midazolam (0.5 mg/kg, up to a maximum dose of 40 mg). The sequence of midazolam administration (i.e., IV followed by oral or oral followed by IV dosing) was determined randomly and separated by a washout period of at least 24 hours. In Arm III, the pharmacokinetics of midazolam and α-hydroxymidazolam were determined after a single IV dose of midazolam (0.15 mg/kg) in patients between the ages of 6 months and < 12 years (Figure1).

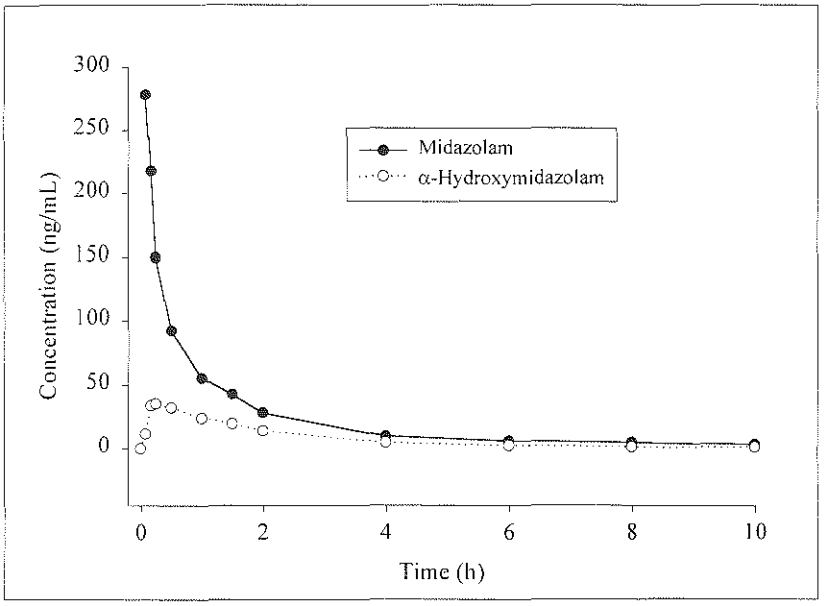

Figure 1 *Mean midazolam and α-hydroxymidazolam plasma concentration-time profile following IV administration of 0.15 mg/kg midazolam to Arm III patients.*

Oral midazolam was administered as a cherry-flavored syrup (Versed® Syrup, Roche Laboratories, Nutley, NJ) in a single swallowing motion whenever possible. After swallowing, each patient was to drink 5 to 10 ml of water to ensure delivery of the dose. Intravenous midazolam was administered by bolus injection over 2 to 3 minutes, after which the infusion line was flushed with an appropriate amount of normal saline to assure complete drug administration.

Blood samples (2 – 2.5 ml) for the determination of midazolam and α-hydroxymidazolam concentrations were obtained at time 0, 10, 20, 30 and 45 minutes and 1, 1.5, 2, 4, 6, 8 and 10 hours after oral syrup administration and at time 0, 5, 10, 15 and 30 minutes and 1, 1.5, 2, 4, 6, 8 and 10 hours after IV administration. In patients whose body weight was < 12 kg, the above sampling strategy was modified, based on the infant's estimated blood volume (e.g., 75 ml blood/kg body weight) to limit potential blood removal to < 3% of the patient's estimated blood volume. All blood samples were obtained from an indwelling venous catheter inserted solely for pharmacokinetic blood sampling and collected in heparinized Vacutainer tubes. Plasma was separated from whole blood by centrifugation (2500 x g for 10 minutes at 4° C) and stored at –70° C until analyzed.

The quantitation of midazolam and α-hydroxymidazolam in plasma was performed using a specific gas chromatography/negative chemical ion mass spectrometry assay. This assay has been validated for both components with acceptable limits of sensitivity, specificity, reproducibility, and linearity.[15,23,24]

Pharmacokinetic Analysis

The disposition of midazolam and α-hydroxymidazolam was characterized by standard noncompartmental pharmacokinetic techniques [25] using the WinNonlin computer software program (Professional Network version 1.5, Scientific Consulting Inc., Cary, NC). The maximum plasma concentration (C_{max}) and time to achieve C_{max} (t_{max}) were determined directly from the individual plasma concentration-time data. The absorption rate constant (K_a) after oral dosing was obtained by fitting the plasma concentration-time data to four different one- and two- compartment WinNonlin models. The results were compared based on visual inspection and the Akaike's Information Criteria (AIC) [26]. The model with the lowest AIC value was chosen for the determination of K_a. The terminal elimination

rate constant (β) was estimated by linear least squares regression analysis of the terminal log-linear portion of the plasma concentration-time curve. The elimination half-life ($t_{1/2}$) was determined as ln 2/β, whereas, the area under the plasma drug concentration-time curve from zero to infinity ($AUC_{0-\infty}$) was determined using the linear trapezoidal rule up to the final measured concentration and extrapolated to infinity using the last measured concentration divided by β. The apparent body clearance (Cl/F), the apparent volume of distribution (V_d/F), the volume of distribution at steady state (V_{dss}) after IV administration, $t_{1/2}$ and $AUC_{0-\infty}$ were calculated by the program. The AUC ratio for α-hydroxymidazolam to midazolam and the absolute bioavailability (F) were calculated according to the following equations:

$$AUC\ Ratio = \left(\frac{AUC_{0-\infty(\alpha-hydroxymidazolam)}}{AUC_{0-\infty(midazolam)}} \right) * \left(\frac{MW_{midazolam}}{MW_{\alpha-hydroxymidazolam}} \right)$$

$$F = \left(\frac{(AUC_{0-\infty})_{PO} * \beta_{PO}}{(AUC_{0-\infty})_{IV} * \beta_{IV}} \right) * \left[\frac{(Dose)_{IV}}{(Dose)_{PO}} \right]$$

where MW is the molecular weight.

For comparison of C_{max} and $AUC_{0-\infty}$ relative to dose administered, C_{max} and $AUC_{0-\infty}$ were dose normalized by dividing the C_{max} and $AUC_{0-\infty}$ by the total midazolam dose administered to each patient.

Midazolam dose proportionality across all age groups in Arm I patients was assessed using the one-way analysis of variance (ANOVA) of the dose-normalized $AUC_{0-\infty}$ and C_{max}. All statistical analyses were performed using standard methods (SAS software version 6.11, SAS Institute Inc., Cary, NC) with the level of significance established at $P < 0.05$.

Two-Stage Population Analysis

A two-stage population analysis was performed by combining oral midazolam data from patients in Arm I, data from patients receiving only IV midazolam from patients in Arms II and III, and historical IV midazolam data sets from Rey et al, [6] Payne et al,[20] and Wells et al.[27] The first stage was the noncompartmental analysis for the estimation of Cl and Cl/F after IV and oral administrations, respectively. The second stage used nonlinear mixed-effect modeling (NONMEM project group, University of California at San

Francisco, program version IV) to simultaneously fit the IV and oral clearances obtained in stage I for the estimation of the population bioavailability and clearance of midazolam across age groups. The baseline mixed effect was modeled by the following equation:

$$Cl_{observed} = Cl * (1-Q) + (Cl/F) * Q + \varepsilon$$

where $Cl_{observed}$ are the observed clearances, Q is zero for IV clearance data or 1 for oral clearance data, Cl and F are the population clearance, and bioavailability estimated by nonlinear regression and ε is the random error.

Proportional interindividual error models for clearance and bioavailability and an additive residual error model were assumed. First order estimates (NONMEM method 1) were calculated to provide individual parameter estimates from the population parameter estimates using Bayesian regression. More complex models incorporating age and weight as covariates were developed, evaluated, and compared to the baseline model. No other covariates were considered. The likelihood ratio test was evaluated when incorporating fixed effects into the population model. Any effect that reduced the minimum objective function (MOF) by more than 3.84 (χ^2, $P < 0.05$; df = 1) was considered significant and remained in the model. The final model only had age as a covariate for Cl:

$$Cl = (\theta_1 * Age + \theta_3) * (1 + \eta_{Cl})$$
$$F = \theta_2 * (1 + \eta_F)$$
$$Cl_{observed} = Cl * (1-Q) + (Cl/F) * Q + \varepsilon$$

where η_{Cl} and η_F are the interindividual errors in clearance and bioavailability, respectively.

7.4 Results

Demographic Characteristics

A total of 133 patients were enrolled in the study: 104 in Arm I, 12 in Arm II, and 17 in Arm III. Twenty-two patients who were randomized did not receive study medication. Overall, 85 patients in Arm I, 10 in Arm II, and 16 in Arm III received midazolam.

Complete blood sampling for pharmacokinetic analysis was available for a total of 87 patients: 67 patients in Arm I, 6 in Arm II, and 14 in Arm III. Six patients enrolled into Arm II were evaluable after both IV and oral drug administration; 1 patient was evaluated after IV administration only, 2 after oral administration only, and 1 patient who received both doses had incomplete data. Reasons that precluded pharmacokinetic assessment in the remaining study patients included lack of cooperation by the patient during the blood sampling process ($n = 15$), loss of IV access ($n = 2$), partial dose administration ($n = 1$), vomiting 45 minutes post dose ($n = 1$), and gastric suction performed 1 hour after oral dosing ($n = 1$).

Demographic and other baseline characteristics for patients who received midazolam are shown in Table I. Since the disposition of midazolam was determined to be linear over the dose range studied , patient demographics and pharmacokinetic results were pooled relative to dose administered and presented by the study age groupings. No patients demonstrated any abnormalities in renal or liver function as evidenced by prestudy laboratory screening . For the two-stage analysis, 32 patients ranging in age from 2 to 9.6 years (mean = 5 years) from historical data and 15 patients with ranging in age from 1 to 15.5 years (mean = 6.4 years) from this investigation were evaluated.

Pharmacokinetic Results

The mean plasma midazolam and α-hydroxymidazolam concentration-time curves relative to dose administered for patients in Arm I are shown in Figure 2. Statistical assessment (ANOVA) of dose normalized midazolam C_{max} ($P = 0.24$) and $AUC_{0-\infty}$ ($P = 0.70$) indicated no significant differences across the 3 different dose groups, independent of age (Table II), confirming the linearity of midazolam over the dose range studied. Dose proportionality was not assessed for α-hydroxymidazolam; pharmacokinetic parameters C_{max} and $AUC_{0-\infty}$ are summarized in Table III (see also Figures 3-4)

.

The single-dose pharmacokinetics after administration of the oral syrup and IV formulations are presented by age group in Tables IV and V for midazolam, respectively. Midazolam absorption was rapid, with adolescents absorbing the drug at nearly half the rate observed in the children aged 2 to <12 years. Consistent with this observation, t_{max} was twice as long in the adolescents compared to patients in the other two age groups (Table IV).

Tabel I Patient Demographics

	Study Arm I by Age Group (n = 67)			Study Arm II by Age Group (n = 6)			Study Arm III by Age Group (n = 14)		
	6 months to < 2 year	**2 year to < 12 year**	**12 year to < 16 year**	**6 months to < 2 year**	**2 year to < 12 year**	**12 year to < 16 year**	**6 months < 2 year**	**2 year to < 12 year**	**12 year to < 16 year**
Number in pharmacokinetic analysis	3	54	10	3	3	0	2	10	2
Sex (male/female)	1/2	33/21	6/4	1/2	2/1		0/2	6/4	1/1
Age (years)	0.7 ± 0.3	6.4 ± 2.6	14.2 ± 1	0.8 ± 0.4	4.9 ± 1.6		1.2	5.3 ± 2.1	15.4 ± 0.2
Weight (kg)	7.8 ± 2.5	25.1 ± 11.4	55.7 ± 10.7	7.4 ± 1.3	18.1 ± 4.2		8.6 ± 0.9	19.5 ± 4.2	62.0 ± 11.3
Height (cm)	68.3 ± 5.7	116 ± 23.2	162.2 ± 12.3	67.7 ± 6.5	103.3 ± 10.4		77.0 ± 2.8	105.3 ± 7.1	162.5 ± 17.7
SCr (µmol/l)	29.7 ± 13.2	37.8 ± 7.0[2]	59.3 ± 12.0	41.0 ± 5.2	41.3 ± 22.1		27	35.2 ± 5.7	48.5 ± 19.1
Bilirubin (µ/l)	12.5 ± 5.3	10.2 ± 4.3[3]	11.0 ± 4.1	6.3 ± 2.6	6.8 ± 1.8		6.8	8.8 ± 3.3	11.2 ± 3.6
Albumin (gm/l)	39.3 ± 0.6	42.4 ± 3.1[3]	43.6 ± 2.9	43.0 ± 9.2	40.7 ± 0.6		44.5 ± 3.5	41.9 ± 1.8	43.5 ± 0.7

Data are presented as the mean ± SD

a. n = 52 (serum creatinine [SCr] not done for 2 patients).

b. n = 51 (total bilirubin and albumin not done for 3 patients).

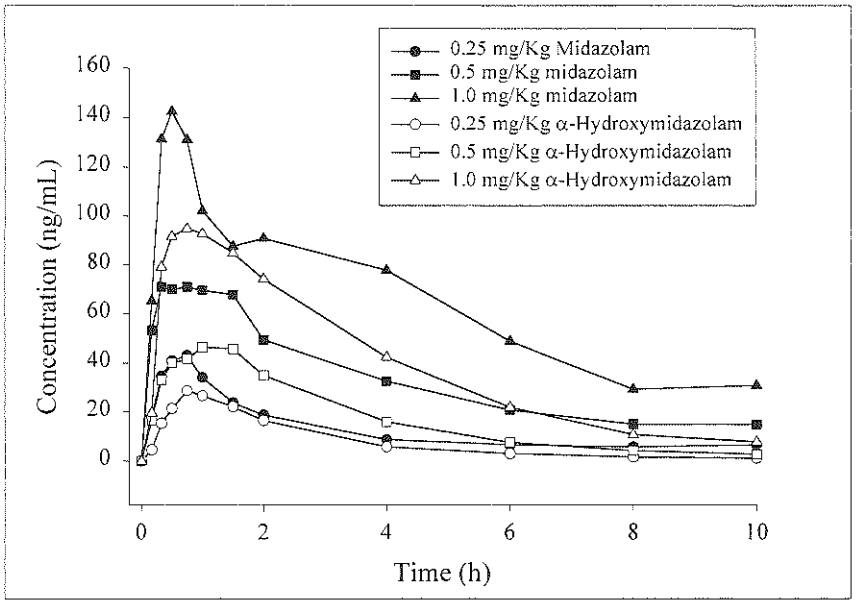

Figure 2. *Mean midazolam and α-hydroxymidazolam plasma concentration-time profile following administration of midazolam syrup to Arm I patients.*

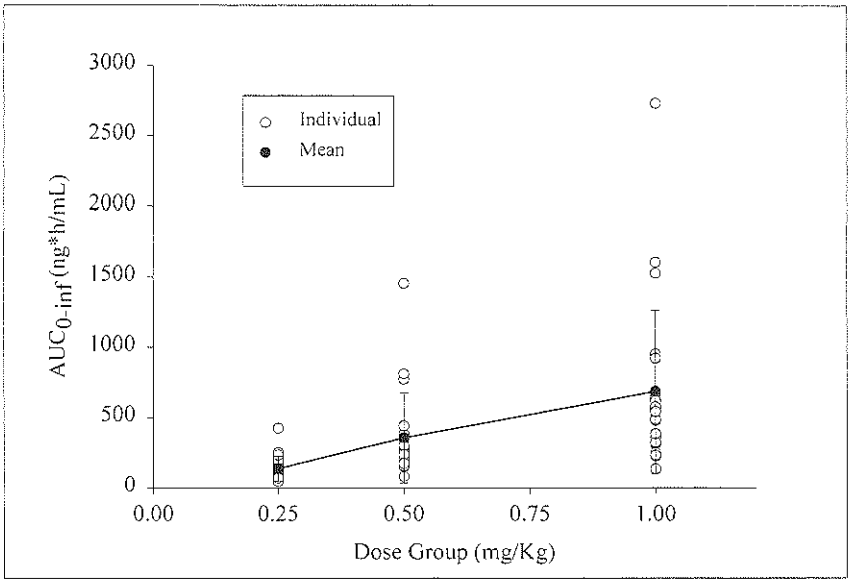

Figure 3. *Midazolam $AUC_{0-\infty}$ versus dose following oral administration of 0.25, 0.50 and 1.0 mg/kg midazolam syrum to Arm I patients.*

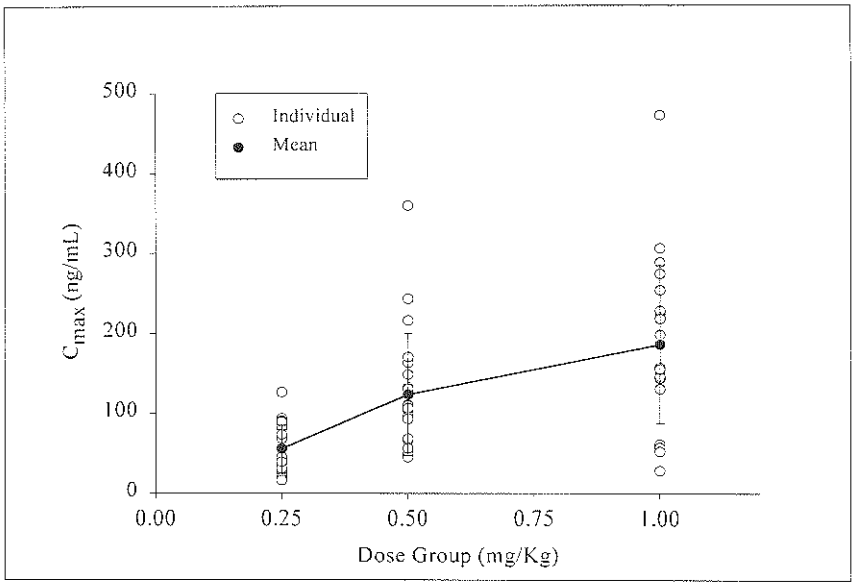

Figure 4. *Midazolam C_{max} versus dose following oral administration of 0.25, 0.50 and 1.0 mg/kg midazolam syrup to Arm I patients.*

Table II Mean Pharmacokinetic Parameters C_{max} and $AUC_{0-\infty}$ for Midazolam following Single Dose Oral Administration of Midazolam Syrup to Arm I Patients

Dose Group (mg/kg)	C_{max} (ng/ml)	Dose-Normalized C_{max} (ng/ml)	$AUC_{0-\infty}$ (ng*h/ml)	Dose Normalized $AUC_{0-\infty}$ (ng*h/ml)
0.25 (*n*=23)	55.6 ± 30.2	10.9 ± 7.6[a]	137 ± 86[b]	23 ± 11[a,b]
0.5 (*n*=21)	123.2 ± 76.1	11.8 ± 10.9	356 ± 320[c]	27 ± 14[c]
0.1 (*n*=23)	185.7 ± 98.6	7.8 ± 4.8	684 ± 581	26 ± 18

Data are presented as the mean ± SD

a. Not statistically different for midazolam across the three dose groups independent of age group.

b. n = 22 (plasma concentration-time profile for 1 patient had no apparent log-linear slope).

c. n = 20 (plasma concentration-time profile for 1 patient had no apparent log-linear slope).

The largest midazolam V_d/F was observed in the youngest age group, whereas midazolam body elimination, as reflected by $t_{1/2}$ and Cl/F was slower in adolescents compared to children in the other 2 age groups. Assessment of these same parameters after IV administration (Table V) revealed only slight differences in V_d across the three age

groups, suggesting the apparent difference observed after oral administration may be primarily due to subject differences in bioavailability. Although less pronounced, the midazolam $t_{1/2}$ was shorter in children < 12 years of age compared to adolescents (12 to < 16 years), although no difference in midazolam Cl/F was observed. Only 2 older patients had complete data following IV administration.

The disposition characteristics of α-hydroxymidazolam after oral and IV midazolam administration are shown in Tables VI and VII, respectively. The greatest formation of metabolite, as reflected by the AUC ratio, was observed in children 2 to < 12 years of age after oral administration. The AUC ratio for patients in the other two age groups (6 months to < 2 years and 12 to < 16 years) were similar. The AUC ratios after IV dosing were similar across age groups and were smaller than the corresponding AUC ratio following oral administration. Elimination ($t_{1/2}$) of α-hydroxymidazolam after oral midazolam administration was more rapid than that of the parent compound; whereas differences in $t_{1/2}$ were not observed after IV administration . The difference in the extent of metabolite formation between oral and IV midazolam administration is likely due to the first-pass metabolism of oral midazolam.

The absolute bioavailability of midazolam in the 6 evaluable patients in Arm III is shown in Table VIII. The midazolam disposition characteristics in these 6 patients after oral administration were similar to the data obtained for all patients in the respective age groups. Overall, the bioavailability of midazolam averaged 36% (CV = 68%), although the range in bioavailability was large (9% to 71%). No relationship between midazolam bioavailability and age was observed, although the study group was small. To better define the relationship between midazolam bioavailability and age, a two-stage population approach was implemented by combining data from patients who received IV midazolam (15 patients from this study [Arms II and III] and 32 historical patients) and patients who received oral midazolam syrup (Arm I). A baseline model was established, which was expanded in complexity to better explain the variability of the population F estimates (NONMEM method 0). Overall, a model including age (over the range of 0.5 to 16 years only) and clearance produced the largest statistically significant reduction in minimum objective function (Table IX). The parameter estimates, standard errors, and 95% confidence intervals of the estimates for the final model with covariates are summarized in Table X. The final population model estimate of midazolam F (36.6%) and the mean of

the individual predictions from the 6 study patients (34.9%) agree closely, although the variability of midazolam F in the population analysis is less than half of that observed in our small study group. The model incorporating age as covariate of clearance suggests that over the age range studied (from 6 months to < 16 years), a roughly threefold change in clearance from 8.7 to 29.3 l/h would be expected.

Table III. Mean Pharmacokinetic Parameters (C_{max} and $AUC_{0-\infty}$) for α-hydroxymidazolam following Single Dose Oral Administration of Midazolam Syrup to Arm I Patients

Dose Group (mg/kg)	C_{max} (ng/ml)	Dose Normalized C_{max} (ng/ml)	$AUC_{0-\infty}$ (ng*h/ml)	Dose Normalized $AUC_{0-\infty}$ (ng*h/ml)
0.25 (*n*=23)	35.6 ± 19.7	7.4 ± 6.0	83 ± 31	15 ± 10
0.5 (*n*=21)	70.3 ± 43.2	6.9 ± 5.2	181 ± 80	16 ± 9
0.1 (*n*=23)	136.6 ± 64.7	5.8 ± 3.3	393 ± 220	16 ± 8

Data are presented as the mean ± SD

Table IV. Disposition Characteristics of Midazolam following Oral Administration of a Single Dose of Midazolam Syrup

Parameter Estimate	Patient Age Group		
	6 months to < 2 years (*n* = 6)[a]	2 years to < 12years (*n* = 57)[b]	12 years to < 16 years (*n* = 10)[c]
K_a (h^{-1})	ND	3.5 ± 2.3[d]	1.3 ± 0.7[e]
T_{max} (h)	0.8 ± 0.8	0.8 ± 0.7	2.0 ± 1.5
$t_{1/2}$ (h)	3.1 ± 1.5	2.7 ± 1.2	4.8 ± 3.3[f]
V_d/F (l/kg)	14.7 ± 10.6	8.6 ± 5.8	9.5 ± 6.0[f]
Cl/F (l/h/kg)	3.0 ± 1.4	2.5 ± 1.9	1.5 ± 0.7[f]

Data presented as the mean ± SD.

a. n = 6 (3 patients from Arm I and 3 patients from Arm II).

b. n = 57 (54 patients from Arm I and 3 patients from Arm II).

c. n = 10 (10 patients from Arm I).

d. n = 23 (number of patients in which K_a could be determined).

e. n = 3 (number of patients in which K_a could be determined)

f. n = 8 (plasma concentration-time profile for 2 patient had no apparent log-linear slope).

Table V Disposition Characteristics of Midazolam following Administration of a Single Intravenous Dose

Parameter Estimate	Patient Age Group		
	6 months to <2 years (n = 5)[a]	2 years to < 12 years (n = 14)[b]	12 years to <16 years (n = 2)[d]
$t_{1/2}$ (h)	2.9 ± 2.2	3.0 ± 1.7	4.5
$AUC_{0-\infty}$ (ng*h/ml)	282 ± 153	281 ± 102	294 ± 122
V_d (l/kg)	2.2 ± 0.5	2.5 ± 1.4	3.6 ± 1.5
V_{dss} (l/kg)	1.2 ± 0.3	1.3 ± 0.7	2.0 ± 0.7
Cl (l/h/kg)	0.68 ± 0.38	0.60 ± 0.23	0.56 ± 0.23

Data presented as the mean ± SD.

a. n = 5 (3 patients from Arm II and 2 patients from Arm III.)

b. n = 14 represents the total number of patients who received IV administration with complete concentration-time profiles (10 from study Arm III, 3 from study Arm II who received both IV and PO, and 1 from study Arm II who received only IV).

c. One patient had a midazolam plasma concentration at time 0.083 hours of 8130 ng/ml, which resulted in an extrapolated C_{max} value of 157,711.1 ng/ml possibly due to an error in the handling of the sample, and was excluded from the analysis.

d. n = 2 (2 patients from Arm III).

Table VI Disposition Characteristics of α-hydroxymidazolam following Oral Administration of a Single Dose of Midazolam Syrup

Parameter Estimate	Patient Age Group		
	6 months to < 2 years (*n* = 6)[a]	2 years to < 12 years (*n* = 57)[b]	12 years to < 16 years (*n* = 10)[c]
T_{max} (h)	1.0 ± 0.7	1.1 ± 0.8	2.2 ± 1.4
$t_{1/2}$ (h)	1.7 ± 0.8	2.0 ± 0.8	3.0 ± 1.6[d]
AUC Ratio	0.54 ± 0.18	0.70 ± 0.30	0.49 ± 0.19[d]

Data presented as the mean ± SD

a. n = 6 (3 patients from Arm I and 3 patients from Arm II).

b. n = 57 (54 patients from Arm I and 3 patients from Arm II).

c. n = 10 (10 patients from Arm I).

d. n = 8 (plasma concentration-time profile for 2 patients had no apparent log-linear slope)

7.5 Discussion

Midazolam is one of the most commonly prescribed benzodiazepines in pediatrics.[3] The drug has consistently been demonstrated to be safe and effective as a sedative, as a premedicant before invasive procedures,[3,5,10-15] and in critically ill infants receiving mechanical ventilation.[3,7-9] More recently, we confirmed the efficacy and safety of midazolam in a large group of pediatric patients who underwent various procedures requiring sedation.[23] Prior to the commercial availability of an oral midazolam syrup formulation, clinicians frequently prepared extemporaneous oral or intranasal preparations using the commercially available IV formulation.[3,12,14,20-22] However, compounding the parenteral formulation with sweeteners or juices to make it palatable was problematic due to the drug's pH-dependent water solubility and the potential for food-drug interactions. The new commercially available cherry-flavored oral midazolam syrup provides a standardized product of high purity, consistent potency, and known stability. The purpose of the present study was to assess the safety, efficacy and pharmacokinetics of this new formulation relative to standard IV administration in a broad age range of pediatric patients after single-dose administration. The efficacy and safety data from this study have been reported elsewhere.[23] The purpose of this report is to describe the overall disposition of IV and oral midazolam in children and adolescents.

The disposition of midazolam was linear over the dose range studied (0.25 – 1 mg/kg) and appeared independent of patient age (6 months to < 16 years). A trend of increasing elimination $t_{½}$ with a corresponding decrease in total (Cl) was observed relative to age, whereas mean residence time (MRT) remained relatively constant across the age groups receiving the same midazolam formulation. Other investigators have described a similar trend in midazolam pharmacokinetics in pediatric patients.[3] The greater amount of α-hydroxymidazolam formed after oral than IV administration is consistent with first pass metabolism of oral midazolam.

Previous studies by Bornemann et al.[19] have shown a greater than proportional increase in midazolam C_{max} and $AUC_{0-\infty}$ with increasing dose from 15 mg to 30 mg in 12 healthy volunteers, presumably due to a transient saturation of the first-pass metabolism of midazolam. Our results in pediatric patients did not indicate a saturation of the first pass metabolism of midazolam to α-hydroxymidazolam for the 0.25 to 1.0 mg/kg oral dose

range. The mean AUC ratio values were 0.54 ± 0.18, 0.70 ± 0.30 and 0.49 ± 0.19 for patients in the 6 month to < 2 years, 2 to < 12 years, and 12 to < 16 years age groups, respectively. The results suggest that patients 2 to < 12 years old have a slightly higher metabolism capacity or slower clearance of α-hydroxymidazolam than adolescent patients or adults[19] (mean AUC ratio range 0.50 to 0.56). The apparent time to metabolite peak concentration in plasma was rapid after administration of both formulations. The α-hydroxymidazolam $t_{½}$ was shorter after oral compared to IV administration. The α-hydroxymidazolam $t_{1/2}$ values are apparent because elimination is dependent on its formation from midazolam.

The pharmacokinetics of midazolam has been extensively studied in a broad range of patient populations and healthy volunteers.[1-3,6-8,18-20,28] Burtin and colleagues[7] described large interindividual variability in midazolam disposition after IV administration in 187 neonates. In their study patients, midazolam Cl and volume of the central compartment were directly proportional to birth weight and midazolam Cl was 1.6 times greater in neonates with a gestational age greater than 39 weeks. Hughes et al,[8] estimating the midazolam Cl from steady-state plasma concentrations in critically ill pediatric patients described a higher midazolam Cl in children > 3 years versus infants and children up to 2 years of age. We did not observe such a relationship in our study patients, although the number of children < 2 years of age was small. Hughes et al[8] also described considerable interindividual variability in the observed steady-state plasma midazolam concentration. A high degree of variability in midazolam disposition characteristics has been described by many investigators [7,8,18,20,27] and was observed in our study. This variability most likely reflects the varying influences that age, diet, sex, disease, ontogeny of drug-metabolizing enzymes, and concurrent medication(s) can have on drug metabolism.[29,30] Midazolam undergoes oxidation via the cytochrome (CYP) P 450 enzyme system. The drug appears to be primarily a substrate for CYP 3A4 but may also be a substrate for CYP 3A5 and 3A7.[3] The overall importance of these later isoforms in postnatal drug metabolism remains to be determined. Substantial interindividual differences in the metabolizing capacity of CYP 2C19, 2D6 and 3A subfamilies have been demonstrated.[29,30] This variability in CYP 3A activity is most likely responsible for the variability observed in midazolam disposition and in midazolam bioavailability after oral administration.

Table VII Disposition Characteristics of α-hydroxymidazolam Following Administration of a Single Intravenous Dose

Parameter Estimate	Patient Age Group		
	6 months to < 2 years (n = 5)[a]	2 years to < 12 years (n = 14)[b]	12 years to < 16 years (n = 2)[c]
T_{max} (h)	0.3 ± 0.2	0.5 ± 0.5	0.9 ± 0.9
C_{max} (ng/ml)	32.5 ± 25.8	32.3 ± 18.5	53.5 ± 53
$t_{1/2}$ (h)	3.2 ± 2.4	2.3 ± 0.9	2.8 ± 0.5
$AUC_{0-\infty}$ (ng*h/ml)	48 ± 11	68 ± 30	128 ± 91
AUC Ratio	0.20 ± 0.11	0.23 ± 0.13[d]	0.39 ± 0.14

Data presented as the mean ± SD.

a. n = 5 (3 patients from Arm II and 2 patients from Arm III).

b. n = 14 represents the total number of patients who received IV administration with complete concentration-time profiles (10 from study Arm III, 3 from study Arm II who received both IV and PO and 1 from study Arm II who received only IV).

c. n = 2 (2 patients from Arm III).

d. One patient had a midazolam plasma concentration at time 0.083 hours of 8130 ng/ml, which resulted in an extrapolated C_{max} value of 157,711.1 ng/ml possibly due to an error in the handling of the sample and was excluded from the analysis.

Table VIII Absolute Bioavailability of Midazolam in Infants and Pediatric Patients Derived From Study Arm II.

	IV Administration (n = 3)		Oral Administration (n = 3)	
Parameter Estimate	**6 months to < 2 years**	**2 years to < 12 years**	**6 months to < 2 years**	**2 years to < 12 years**
β (h^{-1})	0.22 ± 0.1	0.31 ± 0.15	0.3 ± 0.1	0.48 ± 0.2
T_{max} (h)	ND	ND	1.33 ± 0.8	0.7 ± 0.4
C_{max} (ng/ml)	ND	ND	70.7 ± 39.0	67.2 ± 56.5
$t_{1/2}$ (h)	3.9 ± 2.4	2.6 ± 1.0	2.5 ± 0.9	1.8 ± 1.2
V_{darea}/F (l/kg)[a]	2.2 ± 0.7	2.8 ± 2.2	9.4 ± 9.5	14.4 ± 10.9
V_{dss} (l/kg)	1.1 ± 0.3	1.2 ± 0.8	ND	ND
Cl/F (ml/h/kg)[a]	0.4 ± 0.1	0.7 ± 0.3	2.2 ± 1.6	5.9 ± 5.3
$AUC_{0-\infty}$ (ng*h/ml)	372 ± 127	243 ± 110	304 ± 171	190 ± 205
F (%)	100	100	37 ± 21	35 ± 32

Data presented as the mean ± SD

a. F = 1 for IV administration

Unfortunately, the absolute bioavailability of oral midazolam syrup formulation could be estimated on the basis of data from only 6 patients: 3 patients ages 6 months to < 2 years and 3 patients ages 2 years to < 12 years. To address this limitation, a two-stage population analysis was performed to better define the bioavailability of the oral syrup formulation. Combining midazolam concentration-time data from published series[6,20,27] with our complete data set permitted a more critical assessment of the accuracy of the midazolam F estimate determined in our 6 study patients. Overall, the bioavailability of midazolam syrup in these 6 patients averaged 36%, which was nearly identical to the population estimate and compares favorably with previous reports using an oral tablet formulation in adult populations (range: 24 to 49%).[3,18,28] The large variability of midazolam F in this study (9 – 71%) has been observed in other studies[3,18,28] and most likely reflects the ontogeny and variability in intestinal CYP 3A4 activity.[29-31] The two-stage analysis also revealed that the Cl and Cl/F of oral midazolam changed with weight (or age, since age and weight were linearly correlated) after both IV and oral administration, respectively. The weight/age-dependent changes in apparent clearance occur because of the changes in the systemic clearance of midazolam, as seen with the IV doses rather than changes in the absolute bioavailability across the weight/age range.

The midazolam disposition data obtained in the present investigation using the newly available oral syrup formulation of midazolam are consistent with previous reports using extemporaneously prepared formulations. Overall, the disposition characteristics of midazolam and its α-hydroxymidazolam metabolite appeared independent of subject age and dose administered and was linear over the dose range studied (0.25–1 mg/kg). The amount of metabolite formed was greater after oral than IV dosing. Based on the defined absolute bioavailability of the syrup formulation of approximate 36%, oral dosing will need to be increased compared to the routine IV doses employed in clinical practice today. Thus, these data, combined with the drug's long history of overall efficacy and safety over a broad dose range in pediatric patients,[3,23] suggest that an initial oral dose of 0.2 to 0.3 mg/kg should be adequate for the successful sedation of most pediatric patients. Nevertheless, the inherent variability in midazolam intestinal and hepatic metabolism via the CYP 3A subfamily underscores the importance of titrating the midazolam dose to the desired pharmacodynamic effect.

Table IX Predicted Midazolam Clearance and Bioavailability of the Population-Derived and Individual Arm II Analysis

Statistic	Population Model Bioavailability	Arm II Patients Bioavailability
Number of subjects	65[a]	6
Mean	0.349	0.358
Standard Deviation	0.064	0.244
% CV	18.4	68
Minimum Value	0.148	0.086
Maximum Value	0.419	0.711

a. Of the 67 patients who received oral midazolam in study Arm I, 2 patients had no apparent terminal log-linear decline in the concentration versus time plot. Arm II Study patients: see methods for description.

Table X Estimates of Parameters for the Final Model for F and Cl (with Covariates)

Parameter	Estimate	Standard Error of Estimate (CV%)	95% Confidence Interval
θ_1, Proportionality constant of age effect on Cl	1.36	19.5%	0.83-1.89
θ_3, Intercept of age effect on Cl	8.19	16.1%	5.55-10.8
θ_2, F	0.366	10.2%	0.291-0.441
Interindividual variability in Cl	34.4%	34.2%	19.3-44.6%
Intervariability in F	27.8%	26.6%	19.0-34.4%
Residual variability	5.2[a]	65.8%	[b]

a. *Reported as standard deviation.*
b. *The standard error of the estimate is poorly estimated. The 95% confidence interval calculated from this value would include values less than zero and therefore was not reported.*

7.6 Acknowledgment

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Chapter 8

Pharmacokinetics and Metabolism of Intravenous Midazolam in Preterm Infants

based on the article:

Pharmacokinetics and Metabolism of Intravenous Midazolam in Preterm Infants

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Clin Pharmacol Ther 70:525-531 (2001)

8.1 Abstract

Background
Midazolam is a benzodiazepine, finding expanded use in neonatal intensive care units. We studied the pharmacokinetics and metabolism of midazolam after a single intravenous dose in preterm infants.

Methods
The pharmacokinetics of midazolam and its hydroxylated metabolite (1-OH-midazolam) after a single 0.1 mg/kg intravenous dose of midazolam were determined in 24 preterm infants (gestational age, 26 to 34 weeks; postnatal age, 3 to 11 days). Blood samples were obtained before drug administration and at 0.5, 1, 2, 4, 6, 12 and 24 hours after the start of the infusion. Midazolam and 1-OH-midazolam concentrations were determined by gas chromatography-mass spectrometry.

Results
Total body clearance, apparent volume of distribution, and plasma half-life of midazolam were (median [range]): 1.8 (0.7-6.7) ml/kg per min, 1.1 (0.4-4.2) l/kg and 6.3 (2.6-17.7) h, respectively. In 19 out of 24 preterm infants, 1-OH-midazolam concentrations could be detected. 1-OH-midazolam (1-OH-M) maximal concentration of drug in plasma (C_{max}), time to reach C_{max} (T_{max}), and 1-OH-M/M area under the concentration-time curve from time zero to the last sampling time point (AUC_{0-t}) ratio were [median (range)]: 8.2 (< 0.5-68.2) ng/ml, 6 (1-12) h, and 0.09 (< 0.001-1), respectively. Midazolam plasma clearance was increased in those infants who had indomethacin (INN, indometacin) exposure.

Discussion
Consequent to immature hepatic cytochrome P450 3A4 (CYP3A4) activity, midazolam clearance and 1-OH-midazolam concentrations are reduced markedly in preterm infants as compared to concentrations in previous reports from studies in older children and adults. Indomethacin exposure and its apparent impact on midazolam clearance support alteration of drug disposition produced by a patent ductus arteriosus or by the direct effects of indomethacin on hemodynamic or renal function.

8.2 Introduction

Midazolam, a short-acting benzodiazepine, is used for sedation in newborn infants, requiring prolonged mechanical ventilation or need to undergo invasive procedures.[1,2] Despite the use of the agent in neonatal intensive care units, few data are available on its pharmacokinetics in preterm infants < 34 weeks of gestation. Moreover, the data describing the pharmacokinetics of intravenous midazolam in preterm infants show marked interpatient variability.[3]

Midazolam undergoes extensive metabolism by members of the cytochrome P450 3A subfamily (eg, CYP3A4, CYP3A5, and CYP3A7) to a major hydroxylated metabolite (1-OH-midazolam) and several minor metabolites (4-OH, 1,4-OH).[4,5] In adults, plasma clearance of midazolam is correlated significantly with hepatic CYP3A4/5 activity.[6] Cytochrome P450 3A4 (CYP3A4) is the most abundantly expressed cytochrome P450 isoform in adult liver and is responsible for catalyzing the biotransformation of > 50 currently prescribed drugs.[7] Hepatic CYP3A5 expression shows large interindividual differences and displays partially overlapping substrate specificity with CYP3A4. In contrast, CYP3A7 is the major isoform expressed in human fetal liver.[7] Lacroix et al.[8] showed that, irrespective of gestational age at birth, CYP3A4 expression is activated during the first weeks after birth, which is accompanied by a simultaneous decrease in CYP3A7 activity. The developmental pattern of CYP3A5 activity is as yet unknown. As one might expect consequent to the impact of ontogeny on CYP3A activity, midazolam plasma clearance is reduced in critically ill, newborn infants.[3]

Reduced CYP3A4/5 activity after birth will not only reduce midazolam elimination but also the formation of 1-OH-midazolam, an active metabolite.[9] In preterm infants, 1-OH-midazolam after therapeutic midazolam administration demonstrates large interindividual variability.[10] However, the pharmacokinetics of this metabolite in preterm infants has not been determined to date.

To investigate the pharmacokinetics of midazolam and 1-OH-midazolam in the first 2 weeks of life, we evaluated its disposition in preterm infants with gestational ages between 26 and 34 weeks following a single intravenous dose of midazolam.

8.3 Methods

Patient population

The study was conducted in 24 preterm infants; gestational age: 26 to 34 weeks; and postnatal age: 3 to 11 days. The infants were recruited from the neonatal intensive care unit of the Sophia Children's Hospital. All infants received midazolam before a stressful procedure (eg, tracheal tube suction, elective nasopharyngeal intubation) and had a preexisting indwelling arterial catheter placed for purposes of medical care (eg, blood pressure measurements and arterial oxygenation measurements). Patients were excluded if they received morphine, dobutamine, dopamine or a drug known to affect CYP3A activity. In addition, patients were excluded from the study if they had significant underlying hemodynamic, renal, hepatic, or neurologic dysfunction. This research protocol was approved by the Sophia Children's Hospital Human Ethical Committee. Written, informed consent was obtained from parents or legal guardians before enrollment of subjects in the study.

Drug administration and sample collection

Midazolam (Dormicum injection, Roche Laboratories, The Netherlands) was administered as a single 0.1 mg/kg dose in a 5% glucose solution (0.03 mg/ml) infused by syringe pump over 30 minutes through microbore tubing into a peripheral vein or into a central venous catheter. Serial arterial blood samples (0.2 ml) were obtained at baseline and at 0.5,1,2,4,6,12 and 24 hours from the time of dosing. Plasma was separated from whole blood by centrifugation (1000 g for 10 minutes) and then stored at –80°C until analysis. The subjects were observed during the infusion for adverse reactions; vital signs were checked before infusion and at the time of blood samples were obtained.

Analytical methods

Plasma samples were analyzed for midazolam and 1-OH-midazolam by gas chromatography with mass spectrometric detection (Hewlett Packard 6890, Agilent Technologies Inc, Palo Alto, Calif). The column used was a J&W Scientific DB-17 EVDX [0.2 μm, 25 meters (J&W Scientific, Folsom, Calif)]. Benzodiazapine (Diazepam; Elkins Sinn, Cherry Hill, NJ), 5μl of 500 ng/ml solution, was added to each sample as an internal standard and solid phase extraction was performed by using a Varian Bond Elut Column (Varian Inc, Palo Alto, Calif). The interday coefficient of variation for the low

standard (2 ng/ml) was consistently < 10% for both midazolam and 1-OH midazolam. The intraday coefficients of variation were also < 10% for both midazolam and 1-OH midazolam at a concentration of 2 ng/ml. The lower limit of quantitation was 1 ng/ml for midazolam and 0.5 ng/ml for 1-OH-midazolam and 4-OH-midazolam using 0.05 ml plasma. All samples were analyzed in duplicate with the resultant mean concentration was used in the pharmacokinetic analysis.

Pharmacokinetic analysis

The maximal concentration of drug in plasma (C_{max}) and time to reach C_{max} (T_{max}) were determined by visual inspection of the plasma concentration versus time curve. The apparent terminal elimination rate constant (K_e) was estimated by curve fitting with use of a nonlinear, least-squares regression analysis with reciprocal (ie, $1/Y^2$) weighting. Area under the concentration-time curve from time zero to the last sampling time point (AUC_{0-t}) was calculated using the log-linear trapezoidal rule. Extrapolation of the AUC to infinity ($AUC_{0-\infty}$) was calculated by the summation of AUC_{0-t} and C_{pt}/K_e, where C_{pt} represents the plasma concentration at the last sampling time (t) predicted from the fitted terminal elimination curve. The individual $t_{1/2}$ was calculated as $0.693/k_e$. The apparent steady state volume of distribution (V_{area}) and total plasma clearance (Cl) were calculated using standard noncompartmental techniques. Pharmacokinetic parameters of 1-OH-midazolam (with the exception of V_{area} and Cl) were determined as described previously for midazolam. In addition to midazolam clearance, the metabolite-parent AUC_{0-t} ratio was used as a *surrogate* marker of CYP3A activity. All pharmacokinetic analyses were performed by using the Kinetica software package, version 2.0 (Innaphase, Inc, Philadelphia, PA).

Statistical analysis

Results are expressed as means ± SD unless stated otherwise. Because most calculated pharmacokinetic parameters did not show a normal distribution, these are expressed as median (range). Comparison of groups of patients, which were defined according to the following dichotomous covariates: partus (cesarean delivery/spontaneous) feeding (parenteral/enteral), prenatal indomethacin exposure (yes/no), prenatal betamethasone exposure (yes/no), postnatal indomethacin exposure (yes/no), mechanically ventilated (yes/no), caffeine therapy (yes/no) and detectable 1-OH-midazolam concentrations (yes/no), with respect to calculated pharmacokinetic parameters was performed by using

the Mann-Whitney *U* test. Association of continuous covariates (ie, postnatal age, gestational age, postconceptual age, Apgar score) and calculated pharmacokinetic parameters are given as Spearman rank (r_s) correlation coefficients. These statistical analyses were performed by using the SPSS software (version 9.0, SPSS Inc., Chicago, IL). The level of significance accepted for all statistical analysis was $\alpha = 0.05$.

8.4 Results

Clinical results

Twenty-four preterm infants (16 girls, 8 boys) with a median gestational age of 29 weeks (range, 26-34), a median postnatal age of 5.5 days (range, 3-11), and a mean birth weight of 1020 g (range, 760-1630), participated in the study. Thirteen patients were receiving mechanical ventilation, 10 patients received continuous positive airway pressure and 1 patient did not receive ventilatory support. Median (range) fraction of expired oxygen (FiO_2) was 0.21 (range, 0.21-0.29) in patients who received mechanical ventilated and 0.21 (range, 0.21-0.28) in patients who received continuous positive airway pressure by nasopharyngeal tube. Twenty patients were exposed antenatally to indomethacin (to prevent preterm labor) and/or betamethasone (to induce lung maturation). Three patients received both agents and 17 received only betamethasone. Eleven patients were exposed to indomethacin postnatally. Four patients had patent ductus arteriosus, for which they received indomethacin during the study and seven patients had received their last dose of indomethacin at least 24 hours before start of the study. Thirteen patients received caffeine before or during the study for weaning of the ventilatory support or for treatment of neonatal apnea. Antibiotics, in most cases β-lactams and aminoglycosides, were required before or during the study in all patients for suspected or proven infection. Additional drug therapy included surfactant ($n = 17$), morphine ($n = 11$, > 12 h before midazolam administration), and furosemide ($n = 3$). No serious adverse events resulting from midazolam administration were reported throughout the course of the study.

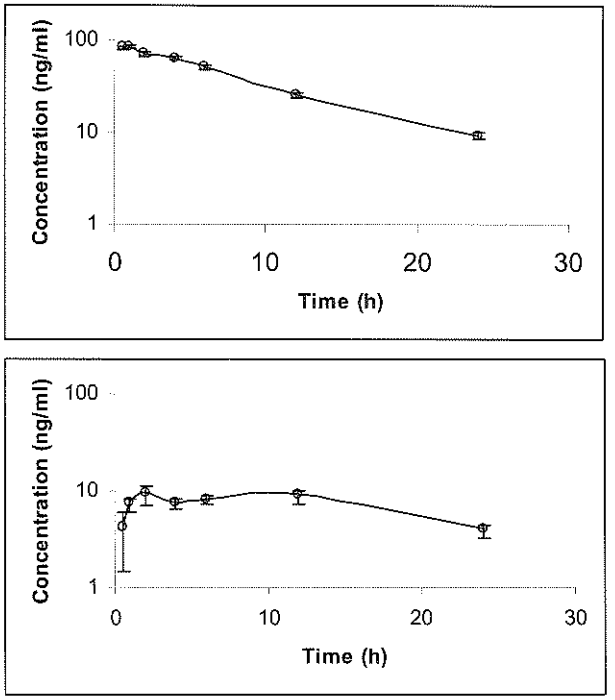

Figure 1. *Intravenous midazolam (A) and 1-OH-midazolam (B) disposition in preterm infants. Midazolam (n = 24) and 1-OH-midazolam (n = 13) concentration versus time log curve after a single intravenous dose (0.1 mg/kg) to preterm infants.Each dot represents mean ± SEM concentration.*

Midazolam and 1-OH-midazolam pharmacokinetics

The mean plasma concentration-time curves for midazolam and 1-OH-midazolam are depicted in Fig 1. Median (range) of midazolam clearance was 1.8 (0.7-6.7) ml/kg/min, volume of distribution was 1.1 (0.4-4.2) l/kg and $t_{1/2}$ was 6.3 (2.6-17.7) h. (Table 1). For 19 of 24 patients, 1-OH-midazolam could be quantitated over the sampling interval. Of these patients, median (range) 1-OH-midazolam C_{max} was 8.2 (< 0.5-68.2) ng/ml with a median T_{max} reached at 6 (1-12) h. The median 1-OH-midazolam AUC_{0-t}/midazolam AUC_{0-t} ratio was low (0.09) and showed large interindividual variation (range, < 0.001–1; coefficient of variation, 191%). No 4-OH-midazolam could be detected.

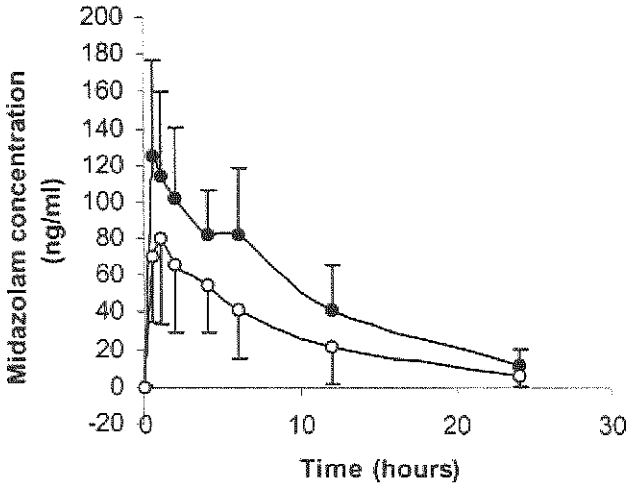

Figure 2 *Effect of postnatal indomethacin exposure on midazolam disposition in preterm infants. Midazolam concentration versus time curve after a single intravenous dose (0.1 mg/kg) to preterm infants with (n = 11, open circles) and without (n = 13, solid circles) postnatal indomethacin exposure. Each dot represents mean ± SD concentration at each time point.*

The effect of covariates on midazolam and 1-OH-midazolam pharmacokinetics

No significant relationship was detected between age (gestational, postnatal, or postconceptional age) and midazolam Cl, V_{area} or $t_{1/2}$. Newborn infants exposed postnatally to indomethacin (n = 11) had a significantly higher mean midazolam clearance as compared to infants who were not exposed to indomethacin (n = 13) (0.17 [range, 0.07-0.40) versus 0.07 (range 0.04-0.24) ml/kg per minute, P = 0.003) (Fig. 2). This effect of indomethacin on midazolam clearance was also found when infants who were exposed to indomethacin but who did not have a patent ductus arteriosus at the time of the study (n = 7) were compared to patients who were not exposed to indomethacin prenatally (mean, 0.14 [range, 0.07-0.40] versus 0.07 [range, 0.04-0.24)] ml/kg per min, P = 0.03). In addition, indomethacin-treated infants had a significantly higher volume of distribution, (mean, 1.7 [range, 0.8-4.2] versus 0.9 [0.4-1.6] l/kg, P = 0.001), whereas mean half-life did not differ between the groups (5.8 [range, 3.9-17.7] versus 6.5 [range, 2.6-14.2] h, P = 0.93). Further, indomethacin-treated infants had a significantly higher postnatal age as compared to nontreated infants (median [range], 6.5 [3-11] days versus 4.5 [3-9] days, P = 0.04). Multiple regression was used to determine which factor was most predictive for midazolam plasma clearance. This analysis showed that midazolam

clearance increased significantly after indomethacin exposure ($P = 0.01$) and when adjusted for this effect, there was no predictive value for postnatal age ($P = 0.43$). We did not find an effect of any of the other clinical parameters (ie, feeding [enteral feeding, $n = 6$], ventilation [mechanically ventilated $n = 13$], Apgar score, partus [spontaneous, $n = 11$], prenatal corticosteroid [$n = 20$], indomethacin administration [$n = 3$], or caffeine therapy ($n = 15$)] on midazolam pharmacokinetic parameters. In addition, there was no relationship detected between 1-OH-midazolam pharmacokinetic parameters (C_{max}, AUC, $t_{1/2}$) and postnatal, postconceptional, or gestational age. No relationship was detected between any of the clinical parameters or concomitant drug therapy and 1-OH-midazolam pharmacokinetics. No statistically significant difference was found with respect to comparison of demographic parameters between patients with or without detectable 1-OH-midazolam concentrations. In those infants for whom 1-OH-midazolam could be quantitated, the 1-OH-midazolam / midazolam AUC $_{0\text{-}t}$ ratio was determined as a surrogate marker for CYP3A activity. No association was observed between any of the demographic parameters and this ratio. Finally, there was not a statistically significant difference in this ratio between patients who were postnatally exposed to indomethacin and those who were not ($AUC_{0\text{-}t}$ ratio: mean, 0.08 [range < 0.001-0.3] versus 0.15 [range, < 0.001-1], $P = 0.90$).

Table 1 Calculated midazolam and 1-OH-midazolam pharmacokinetic parameters in preterm infants

	Midazolam	**1-OH-midazolam**
$AUC_{0\text{-}t}$ (ng/ml.hr)	804 (153 - 2118)	66.7 (< 6 - 997.87)
$AUC_{0\text{-}\infty}$ (ng/ml.hr)	971 (248 – 2353)	76.0 (< 6 -1222.4)
$t_{1/2}$ (h)	6.3 (2.6 – 17.7)	NA
V_{area} (l/kg)	1.1 (0.4 – 4.2)	NA
Cl (ml/kg/min)	1.8 (0.7 – 6.7)	NA
MRT (h)	10.3 (4.0 – 25.6)	NA
C_{max} (ng/ml)	108 (48.8 – 217.0)	8.2 (< 0.5 - 68.2)
T_{max} (h)	0.5 (0.5 – 4.0)	6 (1 - 12)
AUC ratio		0.09 (< 0.001-1)

Data are expressed as median (range), AUC ratio, 1-OH-midazolam$AUC_{0\text{-}t}$/ midazolam$AUC_{0\text{-}t}$, area under the concentration-time curve from time zero to the last sampling time point; $AUC_{0\text{-}\infty}$, area under the concentration-time curve from time zero to infinity; C_{max}, maximal concentration of drug in plasma; Cl, total clearance; MRT, mean residence time; NA, not available; T_{max}, time to reach C_{max}; $t_{1/2}$, elimination half-life; V_{area}, volume of distribution.

8.5 Discussion

For preterm infants, midazolam clearance is lower than previously reported for older children and adults.[11,12] Midazolam plasma clearance (2.3 ± 1.5 ml/kg per minute [± SD]) in our cohort of preterm neonates was comparable to values reported previously in newborn infants with gestational ages between 34 and 41 weeks,[13] but was 1.5 to 5 times lower than those reported in infants older than 3 months (3 - 9 ml/kg per minute), children (5-13 ml/kg per minute), and adults (6-11 ml/kg per minute).[11,14-16] Accordingly, midazolam elimination half-life was longer in our patients (7.6 ± 3.8 h [± SD]) than in older infants, children, and adults (range, 1-2.5 h). This *impaired* midazolam elimination in preterm neonates as compared to that in older infants and children mirrors the known pattern for the ontogeny of CYP3A4.[8] Moreover, although CYP3A7 is also capable of midazolam metabolism, the relative contribution of this substance to the clearance of midazolam in preterm infants is probably only marginal.[4,5] The contribution of CYP3A7 to the metabolism of midazolam may, however, possibly explain part of the large intersubject variability of midazolam clearance in preterm infants.[10]

It is interesting to note that midazolam plasma clearance in our patients was somewhat higher (2.3 ± 1.5 ml/kg per minute[± SD]) than reported by Burtin et al.[3] (1.2 ± 0.96 ml/kg per minute [± SD]) and Lee et al.[10] (1.0 ± 0.2 ml/kg per minute [± SD]). These earlier investigations estimated clearance using population pharmacokinetics in preterm infants with gestational and postnatal ages similar to those of our patients, and who received midazolam as either a continuous infusion or as an intravenous bolus dose. This apparent difference between our mean midazolam clearance and those of earlier studies may be the result of differences in patient population, comedication received, or simply greater variability associated with pharmacokinetic parameter estimation from a population-based approach with sparse sampling. First, only ~ 50% of our infants were ventilated with a relatively low oxygen requirement (FiO_2 range 0.21 –0.29), whereas in the other studies[3,4] all patients were mechanically ventilated (oxygen demand not reported), suggesting that these patients were, on average, less stable than our patients. In adults, midazolam clearance appears to be reduced in critically ill patients, possibly as a consequence of reduced CYP3A activity.[17] Therefore, a difference in disease severity may have contributed to the lower midazolam clearance reported by Lee et al.[10] and Burtin et al.[3] Second, the increasing use of betamethasone over the last few years may also have

contributed to the higher midazolam clearance reported in our patients compared to those in older studies. Twenty out of our 24 patients were exposed to betamethasone antenatally. If betamethasone is capable of inducing CYP3A activity (as is dexamethasone),[18] higher plasma midazolam clearance may have resulted from this particular drug interaction.

Although midazolam elimination in preterms shows age-related differences in relation to infants older than 6 months of age,[11] we did not find a relationship between age (postconceptional, gestational or postnatal) and midazolam clearance or AUC ratio (as a surrogate marker for CYP3A4/5 activity) within our patient group. This finding is in agreement with reports of previous studies of preterm and term newborn infants with gestational ages ranging from 24 to 39 weeks.[10] This lack of relationship between gestational or postnatal age and midazolam elimination mirrors the observation in vitro that CYP3A4 activity increases only marginally during the first 2 weeks of life.[8] However, the lack of a relationship between age and midazolam clearance or AUC ratio in our study should be interpreted with caution given the relatively small sample size ($n = 24$) of our study cohort and the narrow range of gestational (26 to 34 weeks) and postnatal ages (3 to 11 days) that characterized our subjects.

Unexpectedly, postnatal indomethacin exposure, during or at any time before the study, was associated with a higher midazolam plasma clearance and a larger apparent volume of distribution. This may be an effect of altered pharmacokinetics as a result of resolution of a patent ductus arteriosus consequent to indomethacin treatment as has been reported for aminoglycosides, indomethacin and vancomycin.[19] However, most of the patients received indomethacin more than 24 hours before midazolam administration and, on the basis of the clinical data, did not have a patent ductus arteriosus at the time of the study. As shown by van den Anker et al,[20] indomethacin treatment of patent ductus arteriosus in the first days postnatally was associated with a larger apparent volume of distribution of ceftazidime up to two weeks of age. Hemodynamic consequences of the transition from fetal to neonatal circulation with resultant alterations in extracellular fluid dynamics or glomerular filtration may be sufficient to alter midazolam distribution or the clearance of 1-OH-midazolam as reflected by the increased elimination half-life in our patients.

In addition to midazolam clearance, we used the AUC ratio as a surrogate *marker* of CYP3A4/5 activity *in vivo*. A limitation of our study is, however, that we were not able to measure 1-OH and 4-OH-glucuronides in our patients.[21] We originally aimed at measuring also the glucuronides, but because of ethical constraints we were not allowed by the Human Resource Subject Committee to increase the amount of blood sampling to 0.4 ml per sample (totaling 3.2 ml in preterm infants). There is a limitation in blood sampling for performing research studies: 3% of the total blood volume, which for a newborn of 750 g amounts to 1.8 ml total. Consequently, the AUC ratio we calculated is not *corrected* for glucuronidation.

Although 1-OH-midazolam concentrations have been measured in preterm infants,[22,23] the pharmacokinetics of 1-OH-midazolam in preterm infants have, to our knowledge, not been reported previously. The 1-OH-midazolam-midazolam AUC ratio appears to be lower in preterm infants than in older children and adults, consequent to expected developmental reductions in CYP3A4/5 activity (median, 0.09; range, < 0.001-1.0 versus 0.13-0.26).[9,24] However, the 1-OH-midazolam concentration is also dependent on its metabolism rate to 1-OH-midazolam-glucuronide by the uridine diphosphate glucuronosyl transferases. Therefore this low AUC ratio in preterm infants may also reflect developmentally relatively higher 1-OH-midazolam glucuronidation rate than CYP3A mediated 1-OH-midazolam formation rate.

1-OH-midazolam concentrations and AUC ratio showed considerable interpatient variability (Table 1) and in five of our 24 patients no 1-OH-midazolam could be detected. The intersubject variability in the AUC ratio is much larger in our cohort of preterm infants than the variability reported for midazolam and other CYP3A substrates in both pediatric and adult populations.[9] This larger intersubject variability in newborns as compared to the intersubject variation in adults indicates that CYP3A activity in the newborn is certainly as variable as documented in adults, but probably CYP3A activity in the newborn has a larger variability as compared to CYP3A activity in adults. This larger variation may be attributed to the contribution of CYP3A7 to the formation of 1-OH-midazolam. Further, ontogenic variation in glucuronidation activity may contribute to the large intersubject variability in 1-OH-midazolam concentrations and AUC ratios as observed in our preterm infants.

In conclusion, the elimination of midazolam in preterm infants between 26 and 34 weeks gestational age and < 2 weeks of postnatal age is impaired relative to older infants, children, and adults consequent to reduced CYP3A activity. Therefore, midazolam-dosing regimens may need to be altered in young preterm neonates to prevent overdosing attributable to accumulation of midazolam and 1-OH-midazolam with repeated dosing. Part of the large variability in midazolam pharmacokinetics in this neonatal population could be explained by postnatal indomethacin exposure for closure a patent ductus arteriosus and its effects on hemodynamic and renal function.

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Chapter 9

Cytochrome P450 Involvement in the Biotransformation of Cisapride and Racemic Norcisapride *In Vitro*: Differential Activity of Individual Human CYP3A Isoforms

Based on the article:
Cytochrome P450 Involvement in the Biotransformation of Cisapride and Racemic Norcisapride In Vitro: Differential Activity of Individual Human CYP3A Isoforms
Pearce RE, Gotschall RR, Kearns GL, Leeder JS.
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9.1 Abstract

Identification of the human cytochrome P450 (P450) enzymes involved in the metabolism of cisapride and racemic norcisapride [(±)-norcisapride] was investigated at 0.1 and 1 μM, concentrations that span the mean plasma C_{max} for cisapride. Formation of norcisapride (Nor), 3-fluoro-4-hydroxycisapride (3F) and 4-fluoro-2-hydroxycisapride (4F) from cisapride and an uncharacterized metabolite (UNK) from (±)-norcisapride in human liver microsomes (HLMs) were consistent with Michaelis-Menten kinetics for a single enzyme (K_m: 6.0, 14.3, 13.9 and 107 μM; V_{max}: 1350, 696, 568 and 25 pmol/mg protein, respectively). HLMs converted cisapride to Nor at rates that were at least three orders of magnitude greater than those observed for (±)-norcisapride conversion to UNK. The sample-to-sample variation in the rates of Nor, 3F, 4F and UNK formation correlated strongly ($r^2 > 0.796$) with CYP3A4/5 activity in a panel of HLMs ($n = 7$) and was markedly reduced by ketoconazole, a potent CYP3A inhibitor. Ketoconazole virtually eliminated (±)-norcisapride conversion to UNK (94 ± 0.5 %). Studies with 10 cDNA-expressed enzymes revealed that CYP3A4 catalyzed the formation of Nor and 4F at rates >100 times those of non-CYP3A enzymes, and >100- and 50-fold higher than CYP3A5 and CYP3A7, respectively. CYP3A4 was the only P450 capable of UNK formation. Therefore, CYP3A4 is the principal P450 enzyme responsible for the conversion of cisapride to Nor, 3F and 4F and of (±)-norcisapride to UNK. Compared to cisapride, factors related to CYP3A4-mediated (±)-norcisapride metabolism (e.g., ontogeny of drug metabolizing enzymes, inhibition, and induction) should be clinically unimportant due to the apparent lack of dependence on cytochromes P450 for elimination.

9.2 Introduction

Cisapride is a prokinetic drug that acts as a postganglionic serotonin 5-hydroxytryptamine receptor agonist (McCallum et al., 1988). It has been widely used in adults and children for the treatment of gastroparesis and symptoms associated with gastroesophageal reflux disease (Wiseman and Faulds, 1994; Vandenplas, 1998). Cisapride has also been administered frequently to neonates and young infants to facilitate oral feeding and to reduce the potential for severe adverse effects (e.g., apnea and bradycardia) associated with excessive regurgitation of gastric contents (Enriquez et al., 1998; Vandenplas, 1998).

Cisapride is a well tolerated drug with a low incidence (i.e., approximately 2 %) of adverse effects that are predominantly gastrointestinal in nature (Vandenplas et al., 1999). In rare cases, cisapride has been linked to prolonged QT_c intervals and the production of potentially life-threatening ventricular arrhythmias (Lewin et al., 1996; van Haarst et al., 1998). Cardiac side effects have been most commonly reported in individuals receiving accidental overdoses of cisapride or in cases in which cisapride was coadministered with other drugs that alter cisapride clearance (e.g., CYP3A inhibitors) and/or prolong QT_c intervals through effects on the iK_r channel (e.g., azole antifungals, erythromycin, clarithromycin) (Michalets and Williams, 2000). Safety concerns related to the association of these rare, cardiac side effects with cisapride administration prompted a recent, voluntary withdrawal of cisapride from the U.S. market by the manufacturer.

Humans extensively metabolize cisapride to one major metabolite, norcisapride (Nor), and to several minor metabolites, including 3-fluoro-4-hydroxycisapride (3F) and 4-fluoro-2-hydroxycisapride (4F). Norcisapride, which retains the *cis*-conformation present in cisapride, is slowly metabolized further to a metabolite of unknown structure and, to a lesser extent, via oxidation to two lactam metabolites, each with a molecular weight of 327 (Meuldermans et al., 1988a,), as shown in Figure 1. Racemic norcisapride [(±)-norcisapride] possesses approximately one-sixth the prokinetic activity of cisapride but does not appear to be associated with cardiac side effects (Hubbard, 1994; Vandenplas et al., 1999).

The metabolism of cisapride to Nor, 3F and 4F is attributed largely to CYP3A4, although much of the evidence to date has been indirect. Clinical investigations have reported pharmacokinetic interactions between cisapride and compounds known to inhibit CYP3A4 (e.g., erythromycin, clarithromycin, ketoconazole, diltiazem, and grapefruit juice) (Michalets and Williams, 2000). Recent *in vitro* studies (Bohets et al., 2000; Desta et al., 2000) have also attributed the biotransformation of cisapride to CYP3A enzymes. These studies, however, were conducted with concentrations of cisapride (5 and 10 μM, respectively) that greatly exceeded those observed *in vivo* (average plasma C_{max}, 0.17 μM). Therefore, it was uncertain whether the results from the previous *in vitro* studies would reflect cisapride metabolism *in vivo*. Additionally, there was little or no information in the literature regarding the ability of CYP3A5 (a polymorphically expressed CYP3A enzyme present in ~ 25% of adult livers) or of CYP3A7 (a fetal

CYP3A isoform) to catalyze the biotransformation of cisapride, although preliminary data indicate that these two CYP3A isoforms are less active than CYP3A4 in catalyzing cisapride *N*-dealkylation to Nor (Gotschall et al., 1999). Furthermore, there have been no published studies that identify the P450 enzymes involved in (±)-norcisapride metabolism. Given the widespread use of cisapride in the first year of life (Vandenplas et al., 1999) and the switch from fetal CYP3A7 to "adult" CYP3A4 that occurs over the first few weeks of postnatal life (Lacroix et al., 1997), we sought to more fully characterize the role that CYP3A isoforms play in the biotransformation of cisapride to NOR, 3F and 4F and to identify the P450 enzymes involved in the biotransformation of (±)-norcisapride to its metabolites. To achieve these objectives, we conducted *in vitro* studies with cisapride and (±)-norcisapride at concentrations (0.1 and 1.0 μM) that spanned the average plasma C_{max} for cisapride.

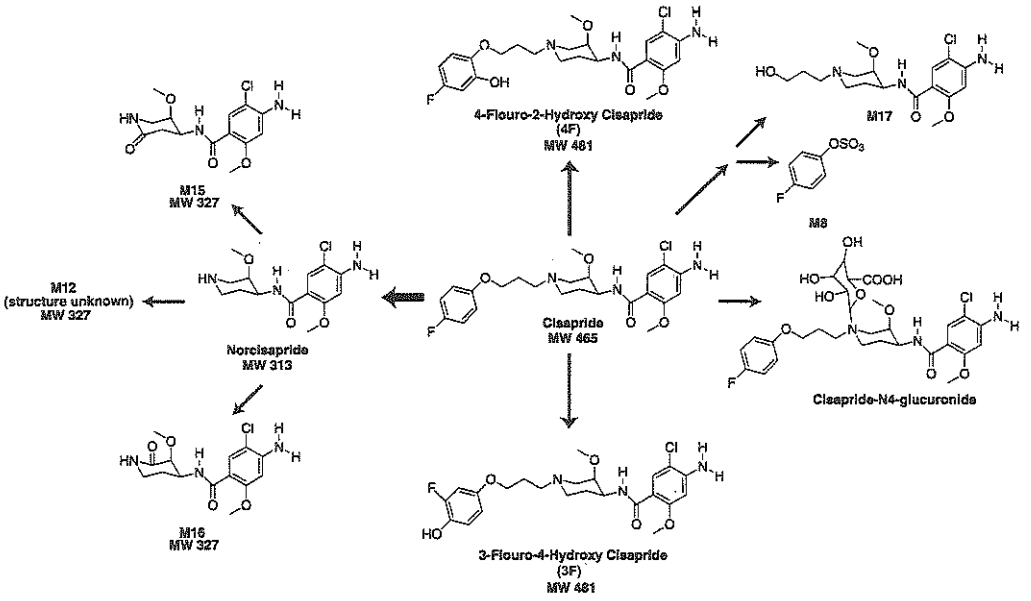

Figure 1. *Structure of cisapride and its major metabolites formed in humans (adapted from Meuldermans et al., 1988a,b)*

9.3 Materials and Methods

Chemicals

Cisapride was purchased from Research Diagnostics (Flanders, NJ). (±)-Norcisapride, 3-fluoro-4-hydroxycisapride, and 4-fluoro-2-hydroxycisapride were kindly provided by Janssen Pharmaceutica (Beerse, Belgium). α-Naphthoflavone, coumarin, (*S*)-(-)-nicotine, orphenadrine, quercetin, sulphafenazole, lansoprazole, quinidine, 4-methylpyrazole, glucose-6-phospate, glucose-6-phosphate dehydrogenase, NADP, and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Ketoconazole was purchased from Sigma/RBI (Natick, MA). All other reagents were of analytical grade.

Human liver microsomes and cDNA-expressed enzymes

Microsomes prepared from seven different human livers were purchased from GENTEST (Woburn, MA). The manufacturer provided data on the sample-to-sample variation of the following P450 enzyme activities: CYP1A2 (phenacetin *O*-deethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2B6 (*S*-mephenytoin *N*-demethylation), CYP2C8 (paclitaxel 6α-hydroxylation), CYP2C9 (diclofenac 4'-hydroxylation), CYP2C19 (*S*-mephenytoin 4'-hydroxylation), CYP2D6 (bufurolol 1'-hydroxylation), CYP2E1 (chlorzoxazone 6-hydroxylation), CYP3A4/5 (testosterone 6β-hydroxylation) and CYP4A9/11 (lauric acid 12-hydroxylation). Microsomes prepared from baculovirus-infected insect cells (SUPERSOMES) expressing human CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP3A7 (coexpressed with human NADPH-cytochrome P450 reductase) were also purchased from GENTEST. Vials of microsomes were stored at -70°C, rapidly thawed in room temperature water, and placed on ice before use.

Incubation conditions

Assay conditions were established for each microsomal sample so that metabolism of the parent compound did not exceed 20 %. Under standard incubation conditions, human liver microsomes (2 or 200 μg of microsomal protein in reactions containing cisapride and (±)-norcisapride, respectively) were incubated at 37 ± 1°C in 100 μl (final volume) incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (1 U/ml), glucose-6-phosphate dehydrogenase (5 mM) and cisapride or (±)-norcisapride (0.1 and 1.0 μM) at the final concentrations listed. *In vitro* enzyme assays were performed in 96-well

microtiter plates. Reactions were initiated by the addition of the NADPH-generating system and terminated by the addition of 200 µl of ice-cold methanol containing 0.15% triethylamine. Incubation mixtures containing (±)-norcisapride were terminated after 120 min, whereas incubation mixtures containing cisapride were terminated after 10 to 40 min, depending on the activity of each microsomal sample. When incubations with microsomes containing cDNA-expressed P450 enzymes were performed, cisapride or (±)-norcisapride (0.1 and 1.0 µM) was incubated with 1 (CYP3A4) or 10 pmol P450 (all other enzymes examined) for 60 min with cisapride as substrate and 20 pmol P450 for 120 min with (±)-norcisapride as substrate. Protein was precipitated by centrifugation at 10,000 rpm for 10 min. An aliquot (10-75 µl) of the supernatant was analyzed by HPLC/MS via direct injection, thereby alleviating the need for an internal standard. The rates of formation of Nor, 3F, and 4F (from cisapride) and of an unidentified product (UNK) (from (±)-norcisapride) were proportional to incubation time and protein concentration (up to 60 min at 0.02 mg/ml protein with cisapride as substrate and up to 120 min at 2 mg/ml liver microsomal protein with (±)-norcisapride as substrate) at substrate concentrations of 0.1 and 1.0 µM. Metabolite formation was also linear in incubations containing 1 (CYP3A4) or 10 pmol P450 (all other enzymes examined) for at least 60 min with cisapride as substrate and 20 pmol P450 for at least 120 min with (±)-norcisapride as substrate. Experiments designed to determine kinetic parameters were performed in duplicate at substrate concentrations ranging from 0 to 100 µM. All other experiments were performed with two replicate samples per condition in triplicate ($n = 6$ determinations) under standard incubation conditions. Rates at which cisapride and (±)-norcisapride were converted to their respective metabolites by recombinant P450 enzymes are reported as background (control) corrected rates.

Chemical-inhibition experiments.

Formation of metabolites from cisapride (0.1 and 1.0 µM) or (±)-norcisapride (1.0 µM) by human liver microsomes was evaluated in the presence or absence (i.e., control) of known P450 isoform-selective inhibitors. The following inhibitors were examined at concentrations previously identified as being appropriate to cause P450 isoform-selective inhibition (Madan et al., 1995; Ko et al., 1997; Stevens et al., 1997; Clarke, 1998; Desai et al., 1998): α-naphthoflavone (CYP1A2; 1 µM), coumarin (CYP2A6; 150 µM), (*S*)-(-)-nicotine (CYP2A6; 200 µM), orphenadrine, (CYP2B6; 500 µM), quercetin (CYP2C8; 20 µM), sulphafenazole (CYP2C9; 10 µM), lansoprazole (CYP2C19; 5 µM), quinidine

(CYP2D6; 0.125 μM), 4-methylpyrazole (CYP2E1; 1 μM), and ketoconazole (CYP3A4/5; 1 μM). Inhibitors were dissolved in methanol and diluted in the incubation mixtures to a final solvent concentration of 1 % (*v/v*). Control incubations contained an equal volume of methanol.

HPLC/MS analysis

Cisapride and its metabolites Nor, 3F and 4F were resolved by reverse-phase HPLC with a Hewlett Packard HP1100 HPLC system equipped with a HP1100 degasser, binary pump, auto-sampler, column heater and a HP1100 single quadrupole mass spectral detector (Hewlett Packard, Palo Alto, CA). The analytical column was a Phenomenex (Torrance, CA) Luna C_{18} (2) column (4.6 mm x 15 cm, 5-μm particle size) preceded by a Phenomenex C_{18} guard column (4-mm x 3-mm i.d., 5-μm particle size). The mobile phase was a 70:30 mixture of methanol/water containing 0.1 % triethylamine. The flow rate was 1 ml/min, and the column temperature was 40°C. The column effluent was monitored using atmospheric pressure chemical ionization detection with a mass spectrometer operating in a selective positive ion-monitoring mode. Ion detection was optimized for Nor, 3F, and 4F detection. The retention times of 3F, Nor, 4F, and cisapride were 2.2, 2.3, 3.2, and 5.1 min, respectively. Data were collected and integrated with Hewlett Packard Chemstation V A.0401 software. Cisapride and its metabolites were quantified by comparison of their peak areas with those of analytical standards. The limit of quantification for the assay was 1 fmol for Nor and 10 fmol for 3F and 4F. The analytical method demonstrated linearity over the range of standard concentrations evaluated, 0.33 to 1000 nM ($r^2 > 0.98$).

(±)-Norcisapride and UNK were measured essentially as described above except that the mobile phase was a 60:40 mixture of methanol/water containing 0.1 % triethylamine. UNK was detected at an *m/z* of 328 (M + 1). Formation of UNK was estimated from the relationship between the amount of (±)-norcisapride analyzed and its peak area, which assumes that (±)-norcisapride and UNK ionize equally under the analysis conditions. It should be emphasized that the method used to quantify UNK provides only a rough approximation of the amounts of UNK analyzed. Under these assumptions, the limit of quantification for the assay was ~ 2.5 fmol for UNK. The retention times of (±)-norcisapride and UNK were 2.3 and 3.1 min, respectively.

Data analysis

Kinetic parameters for the formation of metabolites from cisapride and (±)-norcisapride were estimated from the best-fit line using least-squares linear regression analysis of Lineweaver-Burk plots. Regression coefficients (r^2) between the formation of cisapride or (±)-norcisapride metabolites and the activities of cytochrome P450 enzymes were also determined using least-squares regression analysis. Significance was determined by Pearson's regression analysis from two-tailed *t* tables. To be statistically significant at the 5 or 1 % level of significance, r^2 must exceed 0.448 or 0.764, respectively.

9.4 Results

Metabolism of cisapride and (±)-norcisapride by human liver microsomes

In the presence of NADPH and oxygen, human liver microsomes converted cisapride to Nor, 3F and 4F (based on retention times and positive ion mass of authentic standards). (±)-Norcisapride was converted to one apparent, uncharacterized metabolite (UNK), which was detected at a MS setting of *m/z* 328, suggesting that UNK has a mass of at least 327 Da. In the absence of NADPH or human liver microsomes, no metabolites were formed from either cisapride or (±)-norcisapride.

Correlation experiments

Human liver microsomes prepared from seven donors were examined for their ability to metabolize cisapride and (±)-norcisapride at two substrate concentrations (0.1 and 1.0 μM). All of the microsomal samples examined converted cisapride to Nor, 3F and 4F, and in each case, Nor was the primary metabolite formed. At a substrate concentration of 0.1 μM, the respective rates of Nor, 3F and 4F formation varied ~ 5-fold [range (rates ± S.D.): Nor, 3.78 ± 0.71 to 19.5 ± 1.6 pmol/mg of protein/min; 3F, 2.29 ± 0.71 to 10.3 ± 0.4 pmol/mg of protein/min; and 4F, 1.80 ± 0.65 to 10.0 ± 0.5 pmol/mg protein/min]. Variation in the formation rates for Nor, 3F and 4F among the samples of human liver microsomes was slightly greater (11.7-fold for Nor, range: 11.8 ± 1.9 to 139 ± 27 pmol/mg protein/min; 7.9-fold for 3F, range: 7.77 ± 1.00 to 61.4 ± 3.8 pmol/mg protein/min; and 7.7-fold for 4F, range: 9.24 ± 0.43 to 71.5 ± 15.5 pmol/mg protein/min) at a substrate concentration of 1.0 μM. The rates of Nor, 3F and 4F formation were highly correlated with one another ($r^2 = 0.976$) at both of the substrate concentrations examined. Significant correlations were also observed between the rates of formation of all three

cisapride metabolites with CYP3A4/5 (r^2 = 0.903) and CYP2B6 activities (r^2 = 0.513), but not with any other cytochrome P450 activities.

Microsomes from each of the seven donors converted (±)-norcisapride to UNK. At a substrate concentration of 0.1 μM, the rate of UNK formation varied ~10-fold (0.8 ± 0.1 to 7.7 ± 0.6 fmol/mg protein/min) and was significantly correlated with CYP3A4/5 activity (r^2 = 0.796), but not with any other cytochrome P450 activities. At 1.0 μM (±)-norcisapride, the rate of UNK formation varied ~22-fold (3.3 ± 0.4 to 73.8 ± 1.5 fmol/mg protein/min) and correlated significantly with CYP3A4/5 and CYP2B6 activities (r^2 = 0.887 and 0.606, respectively), but with no other P450 activities. Note that (±)-norcisapride conversion to UNK occurred at rates that were at least three orders of magnitude less than rates of cisapride biotransformation to Nor.

Chemical inhibition of cisapride and (±)-norcisapride metabolism.
The effects of various P450 inhibitors on the conversion of cisapride (0.1 μM) to Nor, 3F and 4F and on the conversion of (±)-norcisapride to UNK (1.0 μM) are illustrated in Figure 2. Because correlation studies implicated CYP3A4/5 as the dominant enzyme involved in the conversion of cisapride to Nor, 3F and 4F, inhibitors were incubated with human liver microsomes from two donors, one with high CYP3A4/5 activity (H112) and one with low CYP3A4/5 activity (H093). Ketoconazole, a potent CYP3A4/5 inhibitor, markedly inhibited the conversion of cisapride (0.1 μM) to Nor, 3F and 4F in liver microsomes with low and high CYP3A4/5 activity. The CYP2B6 inhibitor orphenadrine, had little or no effect on Nor formation; however it did cause modest inhibition of 3F formation in microsomes with high, but not low, CYP3A activity and of 4F formation in microsomes with either low or high CYP3A activity. Quercetin, a reported CYP2C8 inhibitor (Desai et al., 1998), inhibited 3F and 4F formation in microsomes with low and high CYP3A4/5 activity but had little or no effect on Nor formation. Except for modest inhibition of 4F formation by the CYP2C19 inhibitor lansoprazole in microsomes with low CYP3A activity, the other chemicals examined caused little or no inhibition of cisapride conversion to Nor, 3F, or 4F. The P450 inhibitors caused similar effects on the biotransformation of 1.0 μM cisapride to Nor, 3F, and 4F (results not shown) with the following exceptions. First, orphenadrine and quercetin effectively inhibited formation of Nor, 3F, and 4F in both microsomal preparations. Second, sulfaphenazole and lansoprazole moderately inhibited (30-50 %) 3F and 4F formation in both microsomal

preparations, and of Nor formation in microsomes with low CYP3A activity. Interestingly, coumarin appeared to cause a slight increase (up to 40 %) in cisapride (0.1 μM) conversion to Nor, 3F, and 4F, although the reason for this effect is unknown. This effect was not observed at a cisapride concentration of 1.0 μM.

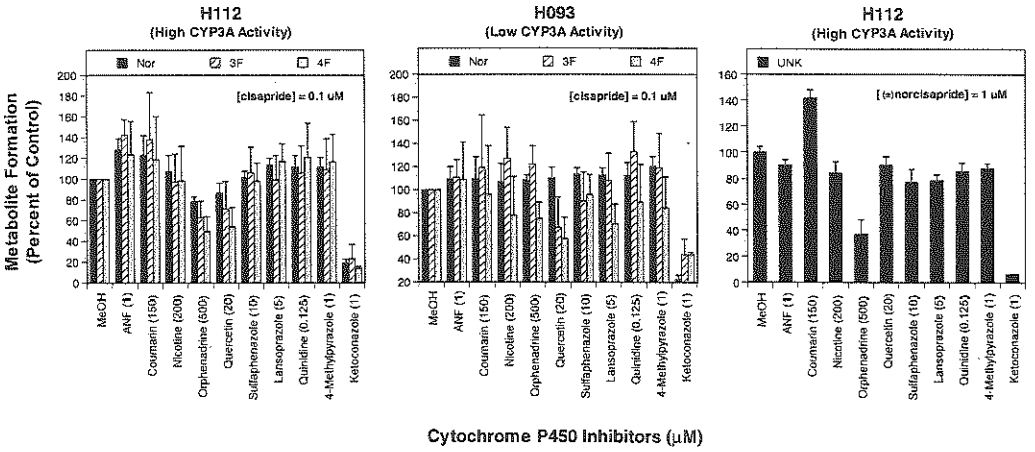

Figure 2. *Effects of various P450 isoform-selective inhibitors on the formation of cisapride and (±)-norcisapride metabolites by human liver microsomes. Human liver microsomes were incubated with cisapride (0.1 μM) or (±)-norcisapride (1.0 μM) in the presence or absence of various chemicals, as described under "Materials and Methods". Final inhibitor concentrations are indicated in the brackets. Each bar represents the mean ± S.D. of six determinations. The uninhibited rates of Nor, 3F, and 4F formation were 139 ± 27, 61.4 ± 3.8 and 71.5 ± 15.5 pmol/mg/min, respectively, in human liver microsomes with high CYP3A activity (H112) and 11.8 ± 1.9, 7.77 ± 1.0 and 9.24 ± 0.43 pmol/mg/min, respectively, in human liver microsomes with low CYP3A activity (H093). The uninhibited rate of UNK formation by human liver microsomes from sample H112 was 73.8 ± 1.5 fmol/mg/min.*

At a concentration of 1 μM, ketoconazole virtually eliminates human liver microsomal CYP3A4/5 activity (Madan et al., 1995). At this concentration, ketoconazole markedly inhibited, but did not eliminate, the formation of Nor, 3F, and 4F from cisapride (0.1 μM), suggesting that 14 to 44 % of the residual activity might be catalyzed by one or more additional P450 enzymes. To investigate this observation further, human liver microsomes from the seven donors were incubated with cisapride (0.1 and 1.0 μM) in the presence or absence of ketoconazole (1 μM), and the results are shown in Figure 3. In the presence of ketoconazole, the residual rate of Nor formation (i.e., the uninhibited rate of (±)-norcisapride formation) correlated significantly with CYP3A4/5 ($r^2 = 0.778$, $P < 0.01$) at both substrate concentrations. Likewise, the residual rate of 3F formation correlated strongly with CYP2C8 activity ($r^2 = 0.721$) at both substrate concentrations but not with the activity of any other cytochrome P450 enzymes. The residual rate of 4F formation was correlated significantly with CYP3A4/5 activity ($r^2 = 0.825$) at both substrate concentrations and with CYP2B6 activity ($r^2 = 0.800$) at a cisapride concentration of 1.0 μM.

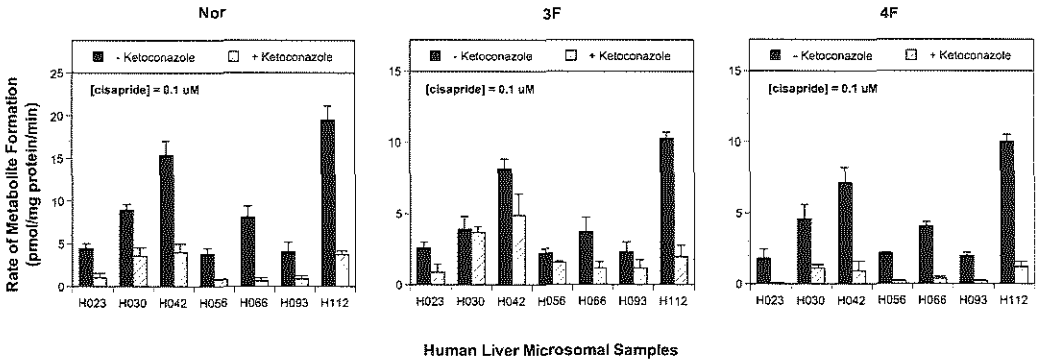

Figure 3. *Effect of ketoconazole (1.0 μM) on the formation of Nor, 3F, and 4F by a panel of human liver microsomes. Human liver microsomes were incubated with cisapride or (±)-norcisapride (0.1 μM) in the presence or absence of ketoconazole (1.0 μM), as described under "Materials and Methods". Each bar represents the mean ± S.D. of six determinations.*

Just as cisapride conversion to Nor, 3F and 4F was significantly correlated with CYP3A4/5 activity, so too was the biotransformation of (±)-norcisapride to UNK. However, the rate at which (±)-norcisapride was converted to UNK by human liver microsomes was at least 2000 times lower than the rate of (±)-norcisapride formation from cisapride. Therefore, to achieve rates of UNK formation sufficient to observe inhibition of (±)-norcisapride conversion to UNK, reactions were conducted at a substrate concentration of 1.0 μM in human liver microsomes with high CYP3A4/5 activity. Under these experimental conditions, ketoconazole virtually eliminated UNK formation. UNK formation was also markedly inhibited by orphenadrine and enhanced by coumarin. The other inhibitors examined had little or no effect on the rate of UNK formation.

Cisapride and (±)-norcisapride metabolism by heterologously expressed human P450 enzymes.

Microsomes prepared from baculovirus-infected insect cells expressing vector alone (control) or 1 of 10 human P450 enzymes were examined for their ability to metabolize cisapride and (±)-norcisapride. Recombinant CYP1A2, CYP2B6, CYP2C8, CYP2C19 CYP3A4, CYP3A5, and CYP3A7 were all capable of forming Nor from cisapride (Table 1), however CYP3A4 was at least 50 times more active at converting cisapride to Nor than the next most active enzyme (CYP3A7). CYP2C9, CYP2D6 and CYP2E1 catalyzed little or no formation of Nor.

CYP3A4 also catalyzed the highest rate of cisapride biotransformation to 3F and was 20 to 50 times more active than the next most active enzyme, which was CYP2C8 (Table 1). CYP3A5, CYP3A7, CYP2C19, and CYP2D6 also converted cisapride to 3F, but at rates substantially lower than those of CYP3A4 or CYP2C8. Cisapride biotransformation to 4F was catalyzed mainly by CYP3A4. Although CYP2C8, CYP3A5, and CYP3A7 also converted cisapride to 4F, they did so at rates that were at least 100-fold less than those of CYP3A4. UNK formation from (±)-norcisapride was solely mediated by CYP3A4 and occurred at rates that were at least 3 orders of magnitude less than rates of cisapride biotransformation to Nor.

Table 1 Conversion of cisapride (0.1 and 1.0 μM) to Nor, 3F and 4F and (±)-norcisapride (0.1 and 1.0 μM) to UNK by heterologously expressed human P450 enzymes.

Expressed Human P450 Enzyme	Metabolite Formation							
	Nor		3F		4F		UNK	
	0.1 μM	*1.0 μM*	*0.1 μM*	*1.0 μM*	*0.1 μM*	*1.0 μM*	*0.1 μM*	*1.0 μM*
	pmol/nmol P450/min							
CYP1A2	< 0.1	0.88 ± 0.06	< 0.1	< 0.1	< 0.1	< 0.1	< 0.005	< 0.005
CYP2B6	0.18 ± 0.02	1.66 ± 0.05	< 0.1	< 0.1	< 0.1	< 0.1	< 0.005	< 0.005
CYP2C8	0.53 ± 0.07	5.38 ± 1.10	1.17 ± 0.54	15.5 ± 2.6	0.14 ± 0.12	1.51 ± 1.31	< 0.005	< 0.005
CYP2C9	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.005	< 0.005
CYP2C19	< 0.1	0.31 ± 0.10	< 0.1	0.57 ± 0.39	< 0.1	0.20 ± 0.15	< 0.005	< 0.005
CYP2D6	< 0.1	< 0.1	< 0.1	0.32 ± 0.19	< 0.1	0.28 ± 0.21	< 0.005	< 0.005
CYP2E1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.005	< 0.005
CYP3A4	130 ± 4	950 ±164	59.8 ± 12.9	341 ± 124	46.8 ± 10.5	338 ± 39	0.092 ± 0.003	0.339 ± 0.019
CYP3A5	0.41 ± 0.03	4.04 ± 0.70	0.19 ± 0.02	190 ± 0.89	0.46 ± 0.10	5.37 ± 2.71	< 0.005	< 0.005
CYP3A7	2.26 ± 0.10	17.6 ± 2.5	<0.1	0.49 ± 0.11	0.11 ± 0.09	1.44 ± 0.94	< 0.005	< 0.005

Table 2. Estimated kinetic parameters for the formation of Nor, 3F and 4F from cisapride and UNK from (±)-norcisapride in human liver microsomes or in heterologously expressed P450 enzymes.
H112 and H093 are human liver microsomal samples with high and low CYP3A4/5 activity, respectively.

	Nor			3F			4F			UNK		
Sample	K_m[a]	V_{max}[b]	V_{max}/K_m[c]	K_m[a]	V_{max}[b]	V_{max}/K_m[c]	K_m[a]	V_{max}[b]	V_{max}/K_m[c]	K_m[a]	V_{max}[b]	V_{max}/K_m[c]
H112	6.00	1350	225	14.3	696	48.7	13.9	568	40.8	107	25.1	0.24
H093	6.90	125	18.1	12.4	78.0	6.29	14.3	67.0	4.68	N.D.	N.D.	N.D.
CYP3A4	3.20	5791	1810	4.30	1628	378	7.90	2480	313	45.5	49.5	1.09
CYP3A7	16.8	203	12.1	18.1	16.0	0.88	25.2	28.7	1.14	N.D.	N.D.	N.D.
CYP2C8	2.00	109	54.5	3.40	289	85.0	5.80	26.7	4.60	N.D.	N.D.	N.D.

N.D., not determined.
[a] *micromolar,*
[b] *Picomoles per milligram of protein per minute for human liver microsomes; picomoles per nanomole of P450 per minute for cDNA-expressed enzymes,*
[c] *Microliters per milligram of protein per minute for human liver microsomes; microliters per nanomole of P450 per minute for cDNA-expressed enzymes*

Determination of kinetic parameters for cisapride and (±)-norcisapride metabolism. Based on the results obtained from the above studies, the kinetics of cisapride metabolism were investigated in human liver microsomes with high and low CYP3A4/5 activity and with recombinant CYP3A4, CYP3A7, and CYP2C8. Kinetic parameters for the formation of UNK from (±)-norcisapride were determined in human liver microsomes with high CYP3A4/5 activity and with CYP3A4.

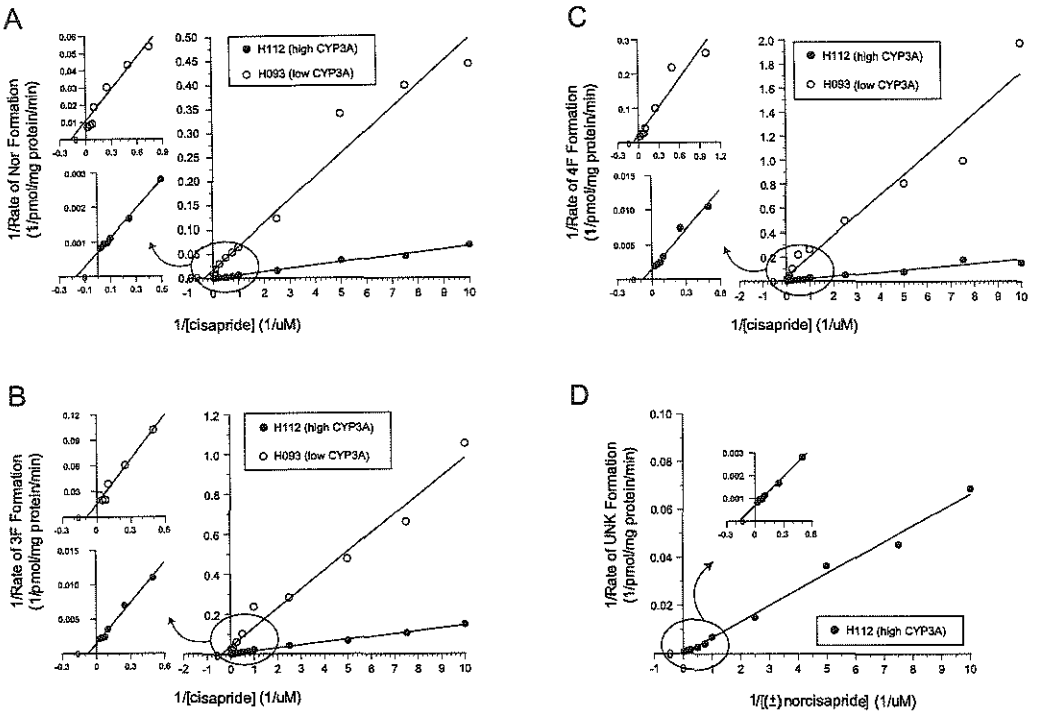

Figure 4. *Lineweaver-Burk (double reciprocal) plots of the effect of substrate concentration on the formation of Nor (A), 3F (B) and 4F (C) from cisapride and UNK (D) from (±)-norcisapride by human liver microsomes. Cisapride and (±)-norcisapride (0-100 μM) were incubated with human liver microsomes for 20 or 120 min, respectively, as described under "Materials and Methods". Each point represents the mean of replicate determinations.*

The formation of Nor, 3F, and 4F from cisapride and of UNK from (±)-norcisapride by human liver microsomes was consistent with Michaelis-Menten kinetics. Lineweaver-Burk plots (Figure 4) for the formation of these metabolites appeared linear over the concentration range examined (0.1-100 μM). This suggests that the formation of each of these metabolites is catalyzed predominantly by a single P450 enzyme (although not necessarily by the same P450 enzyme). The apparent kinetic parameters (K_m, V_{max} and V_{max}/K_m) are contained in Table 2. The V_{max}/K_m ratio, a parameter reflecting *in vitro* intrinsic clearance (Cl_{int}), was ~ 3- to 5-fold higher for the formation of Nor than for the formation of 3F and 4F by human liver microsomes. The Cl_{int} for Nor formation was ~ 1000-fold greater than that of UNK formation from (±)-norcisapride by human liver microsomes.

Formation of Nor, 3F, and 4F by recombinant CYP3A4, CYP3A7, and CYP2C8 and of UNK by CYP3A4 conformed to typical Michaelis Menten kinetics, based on Lineweaver-Burk plots (plots not shown). Kinetic parameters determined from the plots are contained in Table 2. K_m values for the formation of Nor, 3F, and 4F by recombinant CYP3A4 were similar to the corresponding values by CYP2C8, and ~ 3- to 5-fold lower than those by CYP3A7. The Cl_{int} for the formation of Nor, 3F, and 4F by CYP3A4 exceeded that of CYP3A7 by 149-, 430-, and 274-fold, respectively, and that of CYP2C8 by 33.2-, 4.45-, and 68.0-fold, respectively. Note that the K_m of UNK formation from (±)-norcisapride by recombinant CYP3A4 was ~ 14-fold greater than the corresponding K_m of Nor formation from cisapride. The Cl_{int} of Nor formation from cisapride was 1660-fold greater than that of UNK formation from (±)-norcisapride by recombinant CYP3A4.

9.5 Discussion

Consistent with in vivo data demonstrating that Nor is the major metabolite (41 – 45 % of the administered dose) formed from cisapride in humans (Meuldermans et al., 1988b), Nor was the major metabolite formed from cisapride in these in vitro studies. Furthermore, our results indicate that at substrate concentrations that spanned the average plasma C_{max} (0.1 and 1.0 μM), the biotransformation of cisapride is catalyzed principally by CYP3A4. In vitro studies conducted with a panel of human liver microsomes demonstrated that the formation of the major metabolite, Nor, and the minor metabolites,

3F and 4F were significantly correlated with the activity of CYP3A4/5. Ketoconazole, a potent CYP3A inhibitor, markedly reduced formation of Nor, 3F and 4F in human liver microsomes. In human liver microsomes with high CYP3A activity, ketoconazole effectively inhibited ~ 80 – 90 % of cisapride metabolism. Studies with a panel of cDNA-expressed enzymes confirmed that CYP3A4 is a relatively high affinity, high-capacity enzyme capable of converting cisapride to Nor, 3F and 4F with K_m values of ~ 3.2, 4.3 and 7.9 μM, respectively. The specific activity of CYP3A4 for the formation of Nor, 3F, and 4F was at least 50-fold greater than that of the next most active enzyme.

With the possible exception of the CYP2C8-catalyzed formation of 3F in which Cl_{int} mediated by CYP2C8 was ~ 4.5-fold lower than by CYP3A4, other P450s do not appear to contribute significantly to the metabolism of cisapride. Furthermore, if one takes into account the relative abundance of these isoforms (CYP2C8 and CYP2B6 account for ~ 6 and < 1 % (Shimada et al., 1994) of total hepatic P450 content, respectively, whereas CYP3A4 accounts for ~ 30% of total hepatic P450 content), the contribution of CYP2C8 or CYP2B6 to the formation of Nor, 3F, and 4F would be expected to be minimal, especially in individuals with moderate to high constitutive CYP3A activity.

Despite mild to moderate inhibition by the CYP2C9 inhibitor, sulfaphenazole and the CYP2C19 substrate/inhibitor, lansoprazole, CYP2C9 and CYP2C19 do not appear to contribute to the biotransformation of cisapride. In the presence of ketoconazole, which markedly reduced CYP3A activity, the relationship between the formation of cisapride metabolites and either CYP2C9 or CYP2C19 activity was poor. Furthermore, heterologously expressed CYP2C9 and CYP2C19 catalyzed little or no biotransformation of cisapride. It is likely that the inhibitory effects observed with lansoprazole are due to its ability to serve as a substrate for CYP3A4/5 (Pearce et al., 1996), and thus competitively inhibit CYP3A-mediated cisapride metabolism.

These results are qualitatively similar to those previously reported from *in vitro* studies (Bohets et al., 2000; Desta et al., 2000) conducted using higher substrate concentrations that far exceed usual plasma concentrations of cisapride associated with therapeutic administration. Although all three investigations conclude that CYP3A4 is the principal human liver P450 responsible for cisapride *N*-dealkylation, the role of CYP2C8 is less certain. Desta et al., (2000) observed that the CL_{int} for cisapride *N*-dealkylation was 20 –

25 % greater for CYP2C8 compared to CYP3A4. However, after correction for relative abundance of P450 isoforms in human liver microsomes, the principal role for CYP3A4 was apparent. In contrast, the results of this study and that of Bohets et al., (2000) found that the CL_{int} for CYP2C8 was at least 10-fold lower than that observed with CYP3A4. In addition, we observed an approximately eight-fold greater rate of cisapride turnover compared to the previous studies (Bohets et al., 2000; Desta et al., 2000), which may be a function of the experimental conditions (e.g., lower substrate concentrations, lower protein content, and an increased substrate/enzyme ratio) utilized in our *in vitro* incubations.

We report two novel findings regarding the hepatic biotransformation of cisapride and (±)-norcisapride. First, (±)-norcisapride metabolism to an unidentified metabolite, UNK, is minimal, occurring to a very limited extent at the substrate concentrations studied. Under these conditions, UNK formation was almost exclusively CYP3A4/5-mediated and occurred at rates that were at least 3 orders of magnitude less than rates of cisapride biotransformation to Nor. Although the possibility exists that UNK may be a previously unidentified metabolite, it seems more likely that it is one or more of the metabolites described by Meuldermans et al. (1988a,b). We speculate that UNK may correspond to the metabolite of unknown structure, because in other mammalian species, the formation of the two lactam metabolites is catalyzed not by P450 enzymes but by cytoplasmic molybdenum hydroxylases (Lavrijsen et al., 1986).

Second, the other CYP3A enzymes examined (i.e., CYP3A5, an isoform found in ~ 20% of adult human livers, and CYP3A7, the fetal CYP3A isoform) catalyzed rates of metabolite formation that were at least 100- and 50-fold lower, respectively, than those catalyzed by CYP3A4. In addition, at pharmacologically relevant concentrations of cisapride, CYP3A7 appeared to catalyze little or no hydroxylation of cisapride to 3F or 4F. Therefore, CYP3A5 and CYP3A7 do not appear to be capable of catalyzing the biotransformation of cisapride to any appreciable extent.

The results of this study demonstrate that CYP3A4 is the P450 enzyme primarily responsible for the metabolism of cisapride and (±)-norcisapride. CYP3A4 is characterized by marked interindividual variability in both enzyme content and activity (up to 10- and 20-fold, respectively), is the predominant P450 present in adult human

liver (Shimada et al., 1994), and is highly expressed in human small intestine (Thummel et al., 1994). The fetal CYP3A isoform, CYP3A7, accounts for 30 to 50 % of total hepatic P450 content in the fetus. Shortly after birth, activity switches from CYP3A7 to CYP3A4 (Lacroix et al., 1997), although CYP3A7 may be expressed to varying degrees in the adult liver (Schuetz et al., 1994). Livers from infants at 1 month of age possess approximately 30 % of the CYP3A4 activity associated with adult livers. Hepatic CYP3A4 activity in infants appears to approach adult levels of activity by 6 to 12 months of age (Lacroix et al., 1997), and may even exceed adult levels of activity between 1 and 4 years of age based on pharmacokinetic data for known CYP3A substrates (Leeder and Kearns, 1997). Previous clinical data and the *in vitro* results presented here and by others (Bohets et al., 2000; Desta et al., 2000) strongly implicate CYP3A4 as the enzyme responsible for catalyzing the biotransformation of cisapride to its major metabolite, Nor. The results of this study also suggest that CYP3A4 is primarily responsible for the conversion of cisapride to the minor metabolites 3F and 4F. Also, our *in vitro* results demonstrate that CYP3A7 does not catalyze the biotransformation of cisapride to Nor, 3F, or 4F to any appreciable extent. Based on these observations, one would anticipate that the developmental pattern of cisapride biotransformation would reflect CYP3A4 ontogeny, thereby placing the most immature infants at greatest risk for serious cardiac adverse effects associated with an accumulation of cisapride in plasma. However, this anticipated pharmacokinetic pattern was not observed in a study of 37 neonates and young infants who were given cisapride (0.8 mg/kg/day), which produced plasma concentrations following an initial dose similar to those observed in adults taking therapeutic doses of the drug (Kearns et al., 2001). It should be noted, however, that the plasma concentrations of cisapride found in neonates might be affected by differences in the relative contribution of intestinal and hepatic CYP3A isoforms to its metabolism during the first few months of life, particularly since the drug is administered orally. To date the developmental pattern of intestinal CYP3A expression has not been characterized.

In contrast to the situation with cisapride, clinical data and our own *in vitro* studies have shown that the biotransformation of (±)-norcisapride to its metabolite (or metabolites) proceeds extremely slowly. Clinical studies have further shown that (±)-norcisapride is extensively excreted unchanged by renal mechanisms (Meuldermans et al., 1988a). Therefore, factors related to CYP3A4 mediated metabolism (e.g., ontogeny of CYP3A enzymes, inhibition, induction, etc.) will be clinically unimportant with regard to the

disposition of (±)-norcisapride in pediatric or adult patients. It is conceivable that any differences in steady-state plasma concentrations of (±)-norcisapride that occur between neonates and adults may be related to developmental differences in renal drug clearance and/or the apparent volume of distribution for (±)-norcisapride. Thus, the known prokinetic activity of (±)-norcisapride (Hubbard, 1994) and the apparent lack of dependence upon cytochrome P450 enzymes for its biotransformation in man make it a candidate for further development as a therapeutic agent.

9.6 Acknowledgements

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Chapter 10

Cisapride: A Potential Model-Substrate to Assess CYP3A4 Activity

Based on the article:

Cisapride: A Potential Model-Substrate to Assess CYP3A4 Activity

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10.1 Abstract

Introduction
Cisapride was compared to midazolam *in vivo* to determine the potential applicability of cisapride as a CYP3A4 "probe". As well, we evaluated whether cisapride was transported by P-gp.

Methods
Bi-directional transport assays were conducted in LLC-PK1 cells and the derivative cell line L-MDR1 to determine whether cisapride was a substrate for P-gp. A pharmacokinetic study was also conducted in seventeen healthy young adults (n = 8 females) who received intravenous (IV) midazolam (0.025 mg/kg), oral (PO) midazolam (0.15 mg/kg) and oral cisapride (0.07 mg/kg) in a randomized, cross-over design. Plasma concentrations were quantitated from repeated post-dose blood samples (n = 3, 7, and 7 for IV midazolam, PO midazolam and cisapride, respectively) by HPLC with UV detection for midazolam and HPLC/MS/MS detection for cisapride and norcisapride. Pharmacokinetic parameters were determined by non-compartmental methods. Both linear and nonlinear regression techniques were used to examine the association between the apparent plasma clearance of midazolam and cisapride and the cisapride:norcisapride plasma concentrations ratios.

Results
Although not a substrate for P-gp, cisapride inhibited P-gp with an apparent Ki of 16.1 μM. Linear correlations between cisapride Cl and both IV and PO midazolam Cl ($P = 0.01$, $r^2 = 0.43$ and $P = 0.001$, $r^2 = 0.46$, respectively) were found.
Cisapride:norcisapride plasma concentrations ratios at 8 hrs ($P = 0.001$, $r^2 = 0.897$) and 12 hrs ($P = 0.001$, $r^2 = 0.956$) as well as cisapride plasma concentrations at these time points were shown to accurately predict the area under the plasma concentration vs. time curve (AUC) for cisapride.

Conclusions
1. CYP3A4 activity estimates reflected by cisapride Cl/F should be independent of transport by P-gp,
2. concordance between the pharmacokinetics for cisapride and midazolam support the applicability of oral cisapride as a pharmacologic substrate to assess total CYP3A4

activity *in vivo* and

3. cisapride plasma concentrations ratios at 8 and/or 12 hours following a single oral cisapride dose may prove useful as a single-point determination to reflect the AUC and plasma clearance of cisapride and *vide inferre*, total CYP3A4 activity *in vivo*.

10.2 Introduction

The CYP3A subfamily, consisting of the four isoforms (CYP3A4, 3A5, 3A7 and 3A43) is the most abundant of the cytochromes P450 in the human body.[1-3] CYP3A4 accounts for the majority of CYP3A, is present predominantly in the liver[4] and in the intestinal microvilli,[5] and is one of the most extensively studied human cytochromes P450 because of its role in regulating the biotransformation of many xenobiotics and therapeutic drugs.[1,6-8] In humans, CYP3A5 activity is present in up to 60 % of adult liver microsomal samples tested.[9] It is also present in the lung and kidney where it is capable of catalyzing the biotransformation of a number of pharmacologic substrates (e.g., midazolam and cyclosporine).[6] While CYP3A4 is polymorphically expressed, the allelic variants described to date appear to have limited, if any, functional consequences with respect to catalytic activity.[10-14] Finally, large (i.e., 20-fold and higher) interindividual variability in CYP3A expression exists which contributes to substantial variability in the pharmacokinetics of drugs that are substrates for these enzymes.[14-16]

Several pharmacologic probes have been used to assess the activity of CYP3A isoforms.[6,17] Historically, the erythromycin breath test (ERMBT) has been used as the standard method to assess hepatic CYP3A4 activity. However, recent literature has suggested that erythromycin is a substrate for P-glycoprotein (P-gp) and hence, may not solely reflect CYP3A4 activity,[18,19] particularly for orally administered compounds. Discordance between the ERMBT and the disposition of other CYP3A substrates *in vivo* has also been reported.[15,20-21] As well, the ERMBT poses challenges for pediatric use in that it must be administered intravenously and involves the non-diagnostic administration of a radioactive isotope.

Midazolam has been used extensively to assess both hepatic and intestinal CYP3A4/5 activity.[6,22] As reviewed by Streetman et al.,[6] several studies have demonstrated a

significant correlation between midazolam plasma clearance and ERMBT results. Other pharmacokinetic parameters, including ratios of either the area-under-the-curve (AUC) or plasma concentrations for midazolam and its primary CYP3A catalyzed metabolites (e.g., 1'- and 4'-hydroxymidazolam) have also been used as surrogate markers of CYP3A4/5 activity with the ratio of metabolites formed being determined by the relative amounts of each CYP3A enzyme present.[9] Recently, Lin et al.[23] performed a retrospective analysis of 17 adult studies to assess whether a single-point determination of midazolam plasma concentration could accurately predict the AUC and thus, reflect CYP3A activity. They found a significant correlation between the 4-hr midazolam plasma concentration and the AUC following both intravenous ($r^2 = 0.8$) and oral ($r^2 = 0.91$) dosing and hence, demonstrated the utility of midazolam as a pharmacologic probe for total CYP3A (i.e., CYP3A4/5) activity. The relative non-specificity of midazolam for CYP3A4 prompted the authors to suggest that a more selective probe may be needed to assess the activity of CYP3A4 alone. As well, despite the low doses of midazolam used for assessment of CYP3A activity, transient sedation, muscle relaxation, ataxia, and amnesia are commonly observed.[6] Thus, its widespread use as a pharmacologic substrate to assess CYP3A4/5 activity in otherwise healthy pediatric patients is problematic given its pharmacologic effects.[1]

Cisapride is an orally administered prokinetic agent used throughout the world for the treatment of gastroesophageal reflux disease and other gastrointestinal disorders.[24-25] Despite its association with the production of serious cardiac arrhythmias in patients with one or more specific risk factors for this rare adverse event,[26-27] the drug is generally safe and well tolerated.[28] Humans extensively metabolize cisapride to norcisapride (NORCIS) via N-dealkylation (41 – 45 % of the administered dose) and to several minor metabolites.[29] Recent evidence has suggested that the N-dealkylation process is primarily catalyzed by CYP3A4.[30-32] Pearce et al.[32] found that the formation of cisapride to NORCIS and its other minor metabolites at cisapride concentrations that spanned the usual range observed with therapeutic drug administration were consistent with Michaelis-Menten kinetics for a single enzyme. Additionally, CYP3A4 catalyzed the formation of cisapride to NORCIS at rates greater than 100 times that found for non-CYP3A enzymes and more than 100- and 50-fold greater than that observed for CYP3A5 and CYP3A7, respectively. Thus, cisapride biotransformation to NORCIS is primarily the result of CYP3A4 activity and is not significantly impacted by CYP3A5.

Given the apparent selectivity of cisapride biotransformation to reflect CYP3A4 activity in humans, the acceptable adverse event profile for the drug and the fact that it is available in multiple stable oral formulations (e.g., oral tablets and solution), we used a pharmacokinetic approach to examine the utility of cisapride as an *in vivo* pharmacologic "probe" for CYP3A4 by comparing it to midazolam in a population of healthy adults. Since no information regarding the interaction between cisapride and P-gp was available, we evaluated the potential of cisapride to function as a P-gp substrate prior to its *in vivo* evaluation as a specific pharmacologic probe for CYP3A4.

10.3 Methods

P-gp Transport Assays

All experiments were conducted in LLC-PK1 pig kidney cells and the derivative cell line L-MDR1, stably transfected with the human *MDR1* gene prepared by one of the co-authors (E.S.). Cells were seeded onto microporous polycarbonate membrane filters (3.0 μM pore size, 24.5 mm diameter, Transwell 3414, Costar®) at a cell density of 2×10^6 cells per well with 2 ml of cell culture medium on both the apical and basal side of the cell monolayer. The quality of the cell monolayers was determined before each experiment by measuring the transepithelial electrical resistance (TEER) that normally ranged from 200 - 450 Ω^*cm^2. The cells were grown for three days before transport experiments. On the third day the cells were washed, the medium exchanged and the assay initiated by adding cisapride (Janssen Research Foundation, Titusville, NJ) to either the apical or basal compartment at a final concentration of 2 μM. Cells were incubated at 37°C and aliquots (100 - 150 μl) were sampled from the opposite compartment at 1, 2, 3 and 4 hours following the addition of drug substrate. Samples were immediately frozen at -70°C for subsequent analysis by HPLC. Translocation of ^{3}H-vinblastine (VBL) (Moravek Biochemicals, La'Brae, CA) at a concentration of 2 μM was used as a positive control in each experiment.

Inhibition Studies

The inhibition of P-gp by cisapride was characterized by evaluating the uptake of ^{3}H-VBL at varying drug concentrations. Cells in suspension of complete RPMI were transferred to a siliconized microfuge tube and 40 μl of ^{3}H-VBL (0.5 μM) containing the required

concentration of cisapride (0-100 μM) was added at time zero. Cells were incubated at 25°C and uptake stopped at 40 minutes by rapid centrifugation. The supernatant layer was quantitatively removed by aspiration and the cell pellet re-suspended in 0.5 ml of 10 % Triton X-100 (Sigma Chemicals, St.Louis, MO). The cell suspension was subsequently transferred to scintillation vials for radioactivity counting. As above, all experiments were conducted in LLC-PK1 and L-MDR1 cell lines. Additional inhibition experiments to elaborate the ability of cisapride to inhibit P-gp were determined in transwell culture by evaluating the ability of cisapride (20 μM) to inhibit ^{3}H-VBL (2 μM) transport across L-MDR1 monolayers as described above for the transport experiments.

Analytical Methods

The HPLC/MS method previously used to characterize the biotransformation of cisapride *in vitro*[32] was adopted for use in this study. Briefly, samples (30 μl) were spiked with internal standard (R53297, generously supplied by Janssen Research Foundation), extracted into 300 μl of chloroform, the aqueous layer aspirated and the organic phase taken to dryness under vacuum. The samples were subsequently reconstituted in 30 μl of 30:70 dimethyl sulfoxide:mobile phase. A 10 μl aliquot of sample was injected onto a Phenomenex Luna™ C18(2) column (4.6 x 150 mm). The mobile phase consisted of methanol:0.02 % TEA (75:25) at a flow rate of 1 ml/min. Chromatography was performed on a Hewlett Packard HP1100 LC/MS system (Hewlett Packard, Palo Alto, CA) with the eluate monitored using atmospheric pressure chemical ionization interface to a mass spectrometer that was operating in a positive selective ion monitoring mode. All chromatography was performed at 40°C.

External standards were prepared on the day of analysis in drug free cell culture medium. A five point standard curve was used to calculate cisapride concentrations, using the peak area ratio of active compound to internal standard. The analytical method demonstrated linearity over the range of standard concentrations of cisapride (2 to 2000 nmol/l) evaluated ($r^2 > 0.99$) with both intraday and interday assay variability less than 10 % for both analytes at low and high concentrations within the range of linearity. All specimens were analyzed in duplicate and the resultant mean value used for statistical analyses.

10.4 *In Vivo* Study

Study Subjects

The study was conducted in 17 healthy Caucasian adults between the ages of 18 and 50 years. Subjects were recruited by the Clinical Pharmacology Research Center, Bassett Healthcare, Cooperstown, NY, where the clinical phase of the investigation was conducted following approval of the study protocol by the local Institutional Review Board. Subjects were enrolled after written informed consent was obtained. Prior to the study (i.e., within a 4 week window of the first study phase), all subjects underwent a medical history, a complete physical examination and laboratory screening to determine eligibility. A medication history was taken to exclude the recent (i.e., within 30 days) ingestion of drugs and/or natural products known to induce or inhibit CYP3A4 activity. The screening examination consisted of a 12-lead electrocardiogram (to document QTc values in the normal range), a renal panel (i.e., serum chemistries, creatinine, BUN), evaluation of hepatic function (i.e., ALT, AST, GGT), a hematocrit and a urinalysis. These evaluations were repeated for each subject upon conclusion of the study. A urine pregnancy test was performed at screening and prior to each study phase. As well, each subject underwent repeated safety monitoring throughout the study (i.e., performance of vital signs and review of systems at each visit to the study unit) to assess for adverse events.

Drug administration and sample collection

Subjects were randomized to receive each of three study medications using a Latin-square design to insure appropriate counterbalance for statistical analysis. The pharmacologic probe compounds and information pertaining to their administration are as follows:

a. Oral cisapride (Propulsid® Suspension, 1 mg/ml, Janssen Research Foundation, Titusville, NJ), 0.07 mg/kg (therapeutic dose in adults = 0.15 to 0.3 mg/kg) followed by ingestion of 120 ml distilled water at 4°C
b. Oral midazolam (Versed® Syrup, 2 mg/ml, Roche Laboratories, Nutley, NJ), 0.15 mg/kg (therapeutic oral dose in adults = 0.2 to 0.5 mg/kg) followed by ingestion of 120 ml distilled water at 4°C
c. Intravenous midazolam (Versed® for injection, 5 mg/ml, Roche Laboratories), 0.025 mg/kg (therapeutic IV dose in adults = 0.03 to 0.07 mg/kg) given via indwelling peripheral venous catheter over one minute

Between each phase of investigation, a minimum 72-hour "washout" period was exercised to insure that residual concentrations of one pharmacologic probe compound did not influence the disposition of another.

Both the oral solutions of midazolam and cisapride were commercially available and were obtained as a single lot number from the Pharmacy Department of Bassett Healthcare to minimize formulation variations in the content of active ingredient. These drugs were administered in an identical fashion to each subject, which included the immediate post-dose ingestion of a fixed volume of distilled water at room temperature so as to facilitate esophageal drug clearance and minimize variability in gastric emptying. Dosing was performed between 0600 to 0900 hours to minimize any potential diurnal variability in drug disposition. Prior to the oral administration of midazolam or cisapride, all subjects had fasted for an 8 hour pre-dose period. They remained fasting for 2 hours post-dose after which an 8 ounce glass of non-citrus fruit juice was administered, followed at 4 hours post-dose by the resumption of a normal diet consumed at specific intervals (e.g., lunch at 1230-1330 hours, dinner at 1800-1900 hours). For a one-hour period following the administration of each study medication, the subjects remained in the Clinical Pharmacology Research Center in a seated position after which they assumed normal activities. Subjects were instructed not to engage in strenuous exercise for the period of blood sampling on each study day. As well, subjects were prohibited from concomitant use of medications or herbal products that function as substrates, inducers or inhibitors of CYP3A.

Immediately prior to the administration of a study medication, a 21 gauge silastic intravenous cannula (Becton Dickinson, Franklin Lakes, NJ) was inserted into a large vein on the dorsum of the hand or on the forearm and secured in place to provide vascular access for repeated blood sampling. At that time, a pre-dose blood sample (2.5 ml) was obtained. Following administration of cisapride, venous blood samples (2.5 ml each) were obtained at the following post-dose times: 0.5, 1, 3, 5, 8, 12 and 24 hours. After administration of oral midazolam, venous blood samples (2.5 ml each) were obtained at the following post-dose times: 0.5, 1, 2, 3, 4, 6, and 8 hours. Following administration of IV midazolam, venous blood samples were obtained at 0.083, 0.5 and 6 hours based on a previously validated minimized plasma sampling model.[33-34] All blood samples were collected into glass tubes containing anticoagulant (ACD) and were immediately

centrifuged (5,000 x g, 15 min., 4°C) after which, the plasma was removed and placed into 0.5 ml polypropylene vials (VWR, West Chester, PA). Plasma samples were then immediately placed at -70°C where they were stored until analysis; a period not exceeding 12 months from the time of collection.

Vital signs were obtained prior to drug administration and every 30 minutes for 2 hours following midazolam administration. Additionally, after administration of the midazolam doses, all patients received pulse oximetry to monitor for any evidence of oxygen desaturation. All observed and reported adverse events regardless of treatment group or suspected causal relationship to study drug were recorded during the entire period of study.

Analytical Methods

Cisapride and norcisapride were quantitated from plasma (100 µl) using a method validated by Janssen Research Foundation (Beerse, Belgium)[35] who kindly performed the analyses. Briefly, following a solid phase extraction, a 20 µl sample was injected onto the analytical column (C18 BDS-Hypersil, 500 x 4.6 mm, 3 µm, Alltech, Deerfield, IL). The analytes and internal standards (R187847 and R187846, Janssen Research Foundation, Berse, Belgium) were eluted along a gradient (25 % to 90 % organic methanol and 0.1M phosphate buffer) and detected by LC/MS/MS in positive ion-mode (API 3000 Triple Quadrupole Mass Spectrometer, Turboionspray™, MDS Sciex, Concord, Ontario, Canada). The range of linearity for this assay was 1 to 200 ng/ml for both cisapride and NORCIS, and the inter- and intra-assay coefficients of variation were consistently < 10 % for concentrations between 1 and 200 ng/ml. An eight point standard curve using the peak area ratio of active compound to internal standard was used to calculate all cisapride and NORCIS plasma concentrations. The mean plasma concentrations from the analysis of duplicate samples was reported and used to accomplish the pharmacokinetic and statistical analyses.

Plasma midazolam (MDZ) concentrations were determined using a modification of the HPLC method published by Mastey, et al.[36] which used flurazepam as the internal standard. Briefly, 1.0 ml samples of plasma were de-proteinated using acetonitrile, vortexed and placed onto a C_{18} solid-phase preparatory columns (Bond Elut®, Varian, Palo Alto, CA) from which they were eluted with 0.8 ml of 100 % methanol. Samples

were evaporated to dryness under N_2 and reconstituted in 125 µl of a potassium phosphate/acetonitrile/methanol mixture. Chromatographic separation of midazolam was accomplished using a Waters Symmetry C_{18} column (Waters Co., Milford, MA) and a mobile phase consisting of 10 mM potassium phosphate buffer (56 % v/v, pH 7.4) / acetonitrile (26 %) / methanol (18 %). Chromatography was accomplished using a Hewlett Packard 1100 HPLC/UV system (Hewlett Packard Co., Palo Alto, CA) with detection of analytes at 220nm (0.01 AUFS). Using a linear methanol gradient (starting at 15% methanol / 85% mobile phase and ending at 70 % methanol / 30 % mobile phase), approximate retention times for MDZ and flurazepam were 11.6 minutes and 19.7 minutes, respectively. Peak area ratios were used to quantitate midazolam. The method had a range of linearity from 0.25 to 250 ng/ml for MDZ. Inter-assay precision of the method was 9.9 % or less at quality control sample concentrations of 1.0, 10.0, and 100.0 ng/ml. Inter-assay accuracy for the same quality control samples ranged from -5.9 to 9.7 % of nominal values. As with cisapride, all samples for midazolam analyses were performed in duplicate with the mean used for pharmacokinetic analysis. This particular analytical method did not enable quantitation of midazolam metabolites (e.g., 1-hydroxymidazolam, 4-hydroxymidazolam) from plasma samples.

10.5 Pharmacokinetic Data Analysis

The maximal concentration (C_{max}) and time of C_{max} (T_{max}) for both midazolam and cisapride were determined for data from each subject by visual inspection of the plasma concentration versus time curve. The apparent terminal elimination rate constant (λ_z) was then estimated from linear, least-squares regression analysis of the plasma concentration vs. time profile for each analyte. Area under the plasma concentration vs. time curve from time zero to the last sampling time point (AUC_{0-n}) for oral cisapride and midazolam was calculated by using the mixed log-linear rule. The AUC was extrapolated to infinity ($AUC_{0\rightarrow\infty}$) by adding AUC_{0-n} to the area under the tail of each concentration vs. time profile calculated by the following equation: $AUC_{tlast\rightarrow\infty} = C_{tn} / \lambda_z$ where C_{tn} is the plasma concentration at the last sampling point predicted from the fit of the apparent terminal elimination phase. The AUC_{0-n} for IV midazolam was calculated according to the minimal sampling equation, $AUC = 54.4 \times [conc_{30min}] + 814.9 \times [conc_{360\ min}] + 681.8$.[33-34] The apparent total plasma clearance (Cl and Cl/F) was calculated using standard non-

compartmental techniques. Pharmacokinetic parameters for NORCIS were determined using a standard non-compartmental approach. In addition, the parent:metabolite (i.e., cisapride:NORCIS) plasma concentration ratios at each time point and also, for AUC_{0-n} were calculated and used as surrogate markers of CYP3A4 activity. All pharmacokinetic analyses were performed using the Kinetica software package (version 4.0, Innaphase, Inc., Philadelphia, PA). Results from the pharmacokinetic analyses are expressed as mean $\pm$ SD and 95 % confidence limits unless stated otherwise.

10.6 Statistical Analyses

Associations between selected pharmacokinetic parameters for midazolam, cisapride and cisapride:NORCIS plasma concentration ratios were examined using linear regression analysis. As necessary, inverse transformation (i.e., 1/y) was used to evaluate potential associations between plasma drug concentrations and clearance as this is the expected relationship between these covariates. To compare the predictability of single point plasma concentrations of cisapride and the cisapride:NORCIS ratio to predict cisapride AUC, a series of multiple linear regression analyses were conducted. Standard approaches to assess goodness-of-fit (e.g., r^2 values, sum of squared residuals) were used to evaluate all regressions. Statistical analyses were performed using the SPSS software package (version 10.1, SPSS Inc., Chicago, Il) wherein a significance limit of $\alpha = 0.05$ was accepted.

10.7 Results

In vitro study

Cisapride did not show any differences in vectorial transport with respect to apical versus basal translocation. In addition, transcellular transport in L-MDR1 cells was similar to that of LLC-PK1 cells suggesting that cisapride is not a substrate for P-gp (Figure 1). A marked increase in the intracellular accumulation of vinblastine in L-MDR1 cells was observed with increasing concentrations of cisapride (Figure 2).

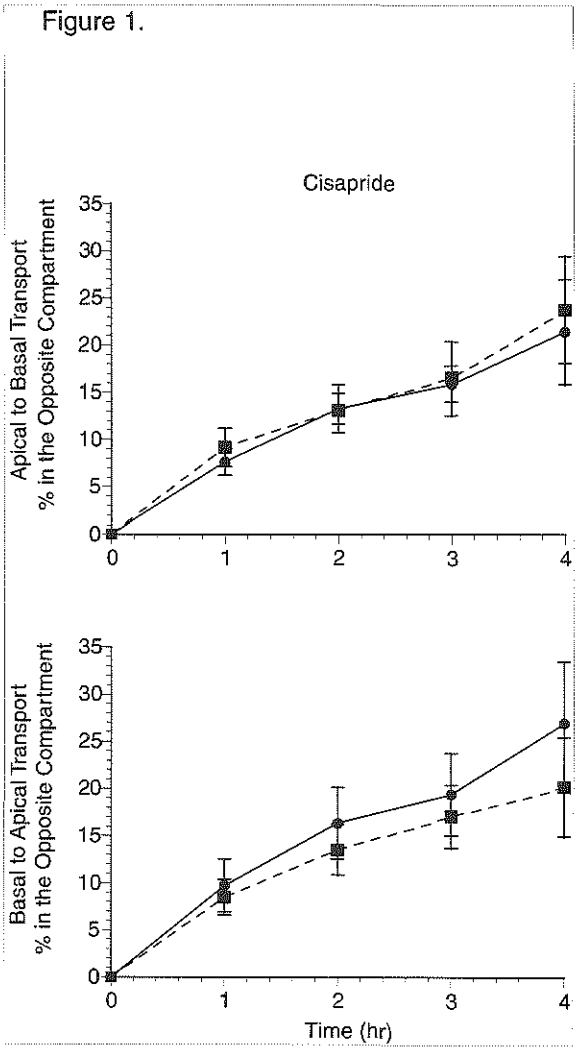

Figure 1. *Transcellular transport of CIS across LLC-PK1 cells (dashed line) and L-MDR1 cells (solid line) from apical to basal compartments (top panels) and basal to apical compartments (bottom panels).*

Figure 2.

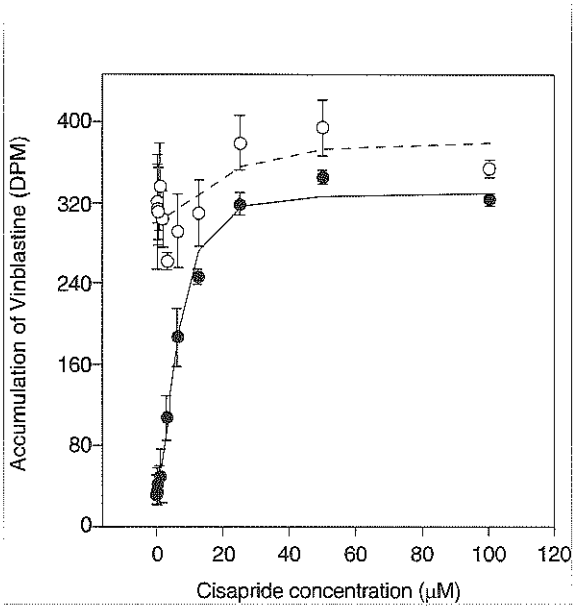

Figure 2 *The effect of CIS on the uptake of ^{3}H-vinblastine in LLC-PK1 cells (dashed line) and L-MDR1 cells (solid lines).*

To determine the relative potency of cisapride as an inhibitor of P-gp, the cisapride concentration required to reverse maximal vinblastine uptake by 50 % (Ki) was calculated from the fit of the mean cisapride vs. vinblastine accumulation plot (Figure 2). This revealed an average value for Ki of approximately 16.1 μM. At concentrations near the Ki (i.e., 20 μM) cisapride significantly inhibited P-gp resulting in an average decrease in vinblastine transport from the basal to apical cell surface by approximately 30 % over baseline and an approximate 3.6-fold increase over baseline in ^{3}H-VBL transport from apical to basal side of the cell (Figure 3).

Figure 3.

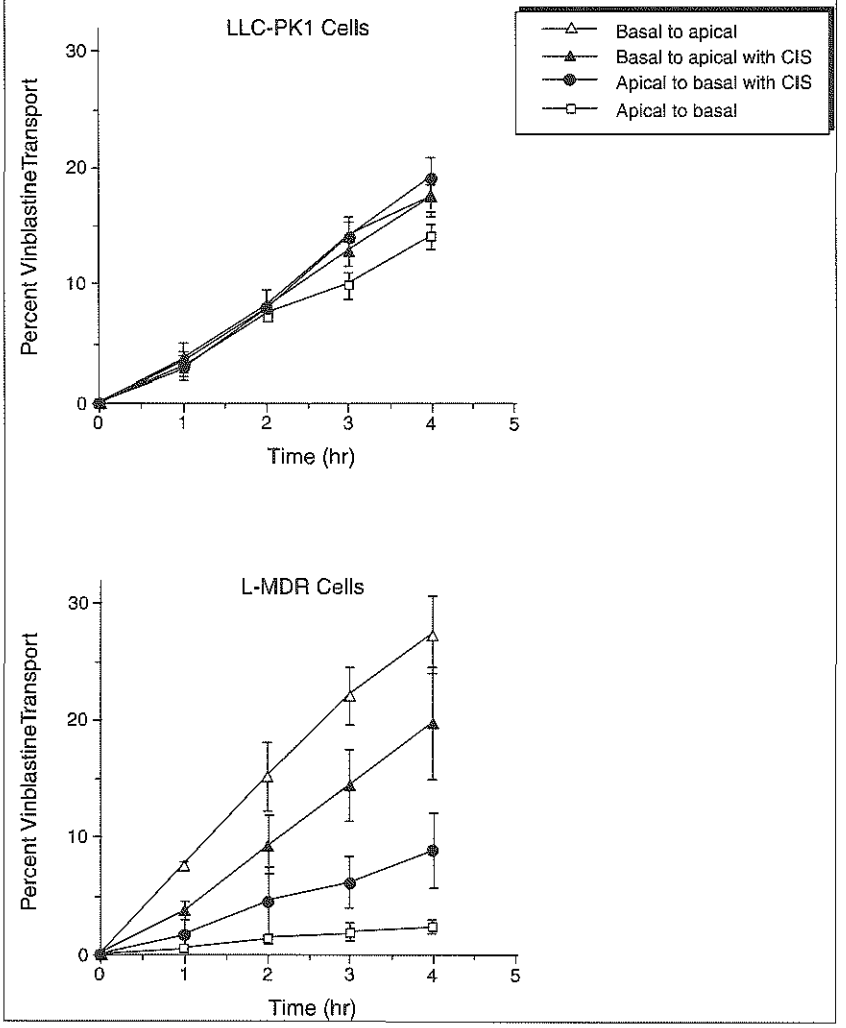

Figure 3 *Vinblastine transport across LLC-PK1 cells (top panel) and L-MDR1 cells (bottom panel) in the presence and absence of CIS.*

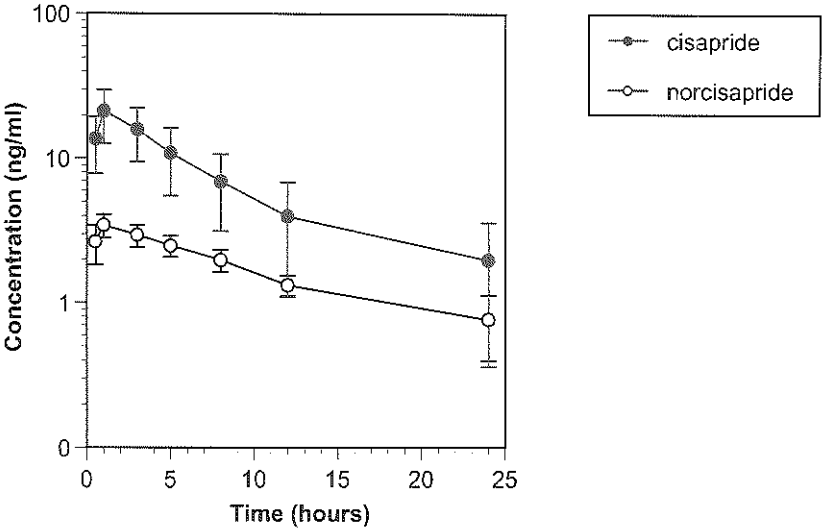

Figure 4 *Concentration vs. time profile (mean ± SD) for cisapride and norcisapride in 17 adults after single dose cisapride oral solution formulation.*

In vivo study

Demographic Data

Seventeen healthy Caucasian adults (9 male, 8 female) completed the study. Their age ranged from 22 to 48 years (38 ± 8 years) and weight from 58.6 to 100 kg (78.0 ± 12.2 kg). Adverse events were noted in the IV and oral midazolam arms of the study. With IV midazolam, the adverse events included drowsiness (n = 2), ataxia (n = 1), hiccups (n = 1), and diarrhea (n = 1). Adverse events noted with oral midazolam were drowsiness (n = 8), vasovagal events (n = 1), ataxia (n = 1), hiccups (n = 1), oxygen desaturation (n = 1), and leg cramps (n = 1). No adverse events were noted during the cisapride arm of the study.

Pharmacokinetic Data

Midazolam could be quantitated in post-dose plasma samples in all subjects through 6 hours after both intravenous and oral administration. All but one subject had detectable plasma concentrations at 8 hours after oral MDZ administration. In all subjects, the apparent time to maximal plasma concentrations (T_{max}) was observed at 0.08 hours and 0.5 to 1 hour for IV and oral MDZ, respectively. Apparent peak plasma concentrations

(C_{max}) were highly variable, ranging from 39.6 to 124 ng/ml (73.05 ± 20.35 ng/ml) and 41.71 to 173.5 ng/ml (97.7 ± 31.43 ng/ml) for IV and oral midazolam, respectively.

Mean plasma concentration vs. time profiles for cisapride and NORCIS are illustrated in Figure 4. Both analytes could be quantitated in all subjects through 12 hours post-dose. Fifteen subjects had detectable cisapride plasma concentrations at 24 hours after drug administration and 11 subjects had detectable NORCIS serum concentrations at 24 hours. In all subjects, T_{max} was observed to occur at 0.5 to 3 hours post-dose for both cisapride and NORCIS. Apparent peak plasma concentrations were variable, ranging from 6.51 to 39.4 ng/ml and 2.32 to 4.7 ng/ml for cisapride and NORCIS, respectively. The mean values for Tmax of cisapride (1.29 hours) and NORCIS (1.26 hours) were virtually identical (Table 1), reflecting rapid biotransformation of the parent drug, presumably at the site of absorption.

The mean (± S.D.) pharmacokinetic parameters for MDZ, cisapride and NORCIS are summarized in Table 1. The average elimination half life for the subjects after oral MDZ dosing (i.e., 2.6 hours) was approximately 1.7-fold higher than observed after intravenous administration (i.e., 1.5 hours) with greater variability observed in the values after the oral (i.e., 1.7 to 3.5 hours) as compared to the intravenous (i.e., 1.2 to 1.7 hours) route. In contrast, similar variability was seen in the $AUC_{0\rightarrow\infty}$ for MDZ following oral and intravenous administration as reflected by the 95 % confidence intervals. As with MDZ, variability was also seen in the elimination half-life (i.e., 1.5-fold), apparent oral clearance (i.e., 1.7-fold) and $AUC_{0\rightarrow\infty}$ (i.e., 1.7-fold) for cisapride as reflected by comparison of the boundaries for the 95 % confidence intervals for each parameter in the study cohort.

Of interest was the finding that the extent of variability in the pharmacokinetic parameters associated with elimination of the model substrates (e.g., elimination T½, Cl, Cl/F) and dose-exposure relationships (i.e., AUC) was dimensionally similar when MDZ was compared to cisapride. This finding suggested the intersubject variability in CYP3A activity (as both compounds are CYP3A4 substrates) in the subject cohort was relatively constant throughout the period of study and thus, not subject to alterations produced by exogenous substances (e.g., inducers or inhibitors) or conditions.

Table 1

	IV Midazolam	Oral Midazolam	Cisapride	Norcisapride
C_{max} (ng/ml)	73.05 ± 20.35	97.7 ± 31.43	21.7 ± 8.3	3.5 ± 0.58
	(62.6 - 83.5)	(81.6 - 113.9)	(17.4 - 26.0)	3.6 (3.24 - 3.84)
T_{max} (hours)	NA	0.62 ± 0.22	1.29 ± 0.83	1.26 ± 0.85
		(0.50 - 0.73)	(0.87 - 1.72)	(0.83 - 1.70)
T½ (hr)	1.46 ± 0.5	2.6 ± 1.7	7.7 ± 2.9	10 ± 2.5
	(1.19 - 1.72)	(1.67 - 3.48)	(6.25 - 9.20)	(8.72 - 11.3)
MRT (hr)	2.1 ± 0.72	3.7 ± 2.4	11.2 ± 4.1	13.5 ± 2.2
	(1.72 - 2.49)	(2.41 - 5.02)	(9.02 - 13.3)	(12.3 - 14.6)
Cl (l/hr/kg)	0.23 ± 0.07	NA	NA	NA
	(0.19 - 0.27)			
Cl (l/hr/kg)/F	NA	0.72 ± 0.38	0.51 ± 0.25	NA
		(0.53 - 0.92)	(0.38 - 0.64)	
AUC_{0-n} (ng*hr/ml)	114 ± 37.4	224.3 ± 86.7	154 ± 77.2	36.1 ± 9.1
	(94.8 - 133.2)	(179.7 - 268.9)	(114.3 - 193.6)	(31.4 - 40.8)
$AUC_{0-\infty}$	123.6 ± 46.9	251.8 ± 102.1	173 ± 90.6	47 ± 9.2
(ng*hr/ml)	(99.4 - 147.7)	(199.3 - 304.3)	(126.4 - 219.6)	(42.2 - 51.7)

Data are expressed as mean ± 1 SD (95% Confidence Interval). Abbreviations include: C_{max}, maximal concentration of drug in plasma; T_{max}, time to maximal concentration; T½, elimination half-life; MRT, mean residence time; Cl, total clearance; F, absolute bioavailability; AUC_{0-n}, area under the concentration time curve from time zero to the last sampling point; $AUC_{0-\infty}$, area under the concentration time curve from time zero to infinity; and NA, not applicable.

The mean elimination T½ for NORCIS (10 hours) was approximately 30 % greater than that for cisapride (7.7 hours) although these values were not significantly different. As expected, the mean C_{max} and $AUC_{0\rightarrow\infty}$ for NORCIS were significantly lower than the values of these parameters for cisapride (i.e., 16 and 27 % of corresponding values, respectively) suggesting that the extent of biotransformation *in vivo* was less than 30 %. Finally, the absolute bioavailability (F) of MDZ in the study cohort was estimated by comparing the mean values for $AUC_{0\rightarrow\infty}$ corrected for dose (i.e., per 1 mg/kg dose) following oral (i.e., 1,720 ng*hr/ml) and intravenous (i.e., 4,944 ng*hr/ml) using the following equation: $F = AUC_{0\rightarrow\infty\ po} / AUC_{0\rightarrow\infty\ iv}$ which yielded a value of 0.35 (i.e., 35 %).

figure 5 a

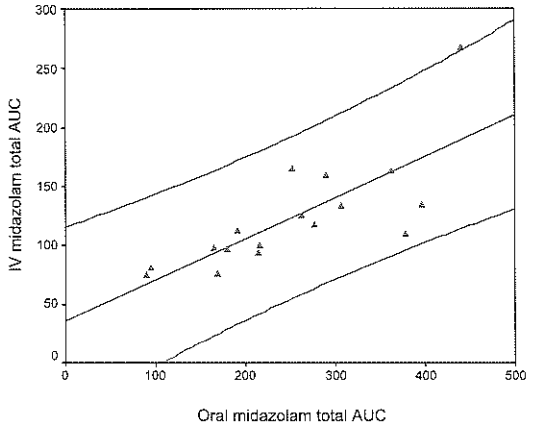

figure 5 b

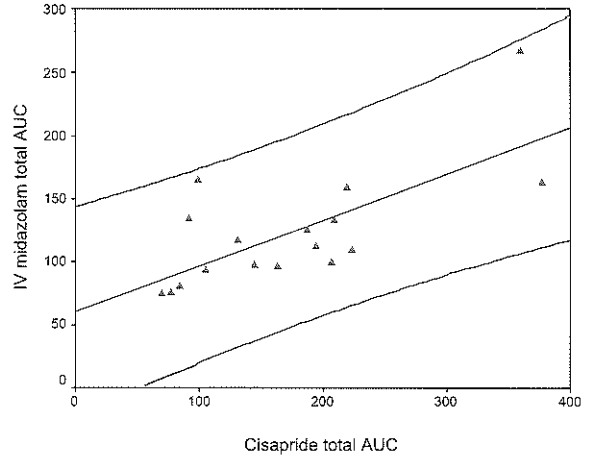

Figure 5 *Panel a: linear correlation (with 95 % CI) between IV and oral midazolam (r^2 = 0.58, P < 0.001). Panel b.: linear correlation (with 95 % CI) between IV midazolam and oral cisapride (r^2 = 0.49, P = 0.002).*

Midazolam and Cisapride Correlations

A linear correlation ($r^2 = 0.58$, $P < 0.001$) was observed between the $AUC_{0\rightarrow\infty}$ for IV and oral MDZ (Figure 5a). The $AUC_{0\rightarrow\infty}$following IV MDZ was also significantly correlated with cisapride $AUC_{0\rightarrow\infty}$ ($r^2 = 0.49$, $P = 0.002$; Figure 5b) as was the $AUC_{0\rightarrow\infty}$ for oral midazolam and cisapride ($r^2 = 0.43$, $P = 0.004$). As expected, significant linear correlations ($r^2 = 0.63$, $p < 0.001$) for clearance values between IV (Cl) and oral

midazolam (Cl/F) were found. As well, the Cl/F for cisapride was positively correlated with the apparent plasma clearance of both IV and oral midazolam ($r^2 = 0.47$, $P = 0.002$; and $r^2 = 0.46$, $P = 0.003$; respectively; Figures 6a and 6b).

To assess the reliability with which cisapride $AUC_{0\rightarrow\infty}$ and Cl/F could be predicted using limited plasma sampling data, we evaluated both single cisapride plasma concentrations and cisapride:NORCIS plasma concentration ratios at times (i.e., concentration at time = t_X) corresponding to the respective post-dose plasma concentration measurements. Cisapride plasma concentrations at 8 and 12 hours were shown to be highly predictive of both cisapride Cl/F (inverse relationship, $r^2 = 0.946$, $P < 0.001$ and $r^2 = 0.725$, $P < 0.001$, respectively) and $AUC_{0\rightarrow\infty}$ (linear relationship, $r^2 = 0.959$, $P < 0.001$ and $r^2 = 0.944$, $P < 0.001$, respectively; Figure 7). Cisapride:NORCIS plasma concentration ratios at 8 and 12 hrs were also shown to reliably predict cisapride Cl/F (inverse relationship, $r^2 = 0.804$, $P < 0.001$ and $r^2 = 0.912$, $P < 0.001$, respectively) and $AUC_{0\rightarrow\infty}$ (linear relationship, $r^2 = 0.897$, $P < 0.001$ and $r^2 = 0.956$, $P < 0.001$, respectively). Use of a multiple linear regression algorithm to examine the addition of a second time point to the correlation analysis did not substantially improve the ability of cisapride plasma concentrations or the CIS:NORCIS ratio to predict either cisapride Cl/F or AUC (data not shown).

10.8 Discussion

Our data indicate that the assessment of cisapride pharmacokinetics following a single, small oral dose may provide a reliable and accurate *in vivo* assessment of combined hepatic and intestinal CYP3A4 activity. As denoted previously by us and others,[1,6,23] selectivity for the enzyme is an absolute requirement for the selection of a pharmacologic probe substrate to assess CYP3A4 activity. On the basis of its biotransformation, CYP3A4[31,32,37,38] (in contrast to CYP3A5 or CYP3A7) appears to be quantitatively and qualitatively responsible for the majority of cisapride metabolism in humans. This is not the case for midazolam which is also a substrate for CYP3A5.[39] Furthermore, Pearce et al.[32] have demonstrated that the dependence of cisapride biotransformation by CYP3A4 occurs at cisapride concentrations similar to those observed in plasma following therapeutic administration of the drug; another important concern when considering its use as a pharmacologic probe to assess activity of the enzyme *in vivo*.

figure 6a

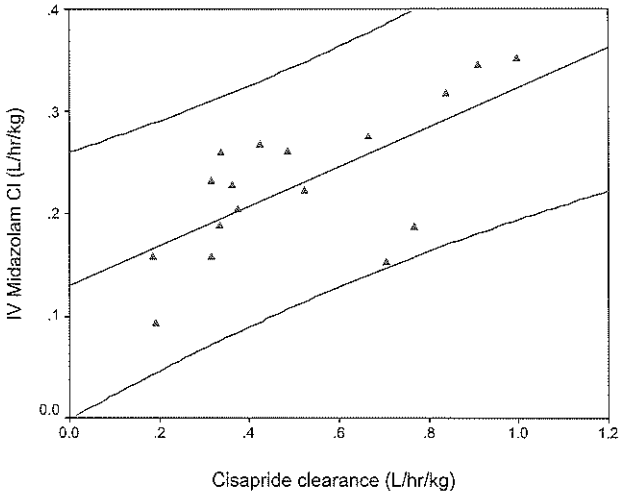

figure 6b

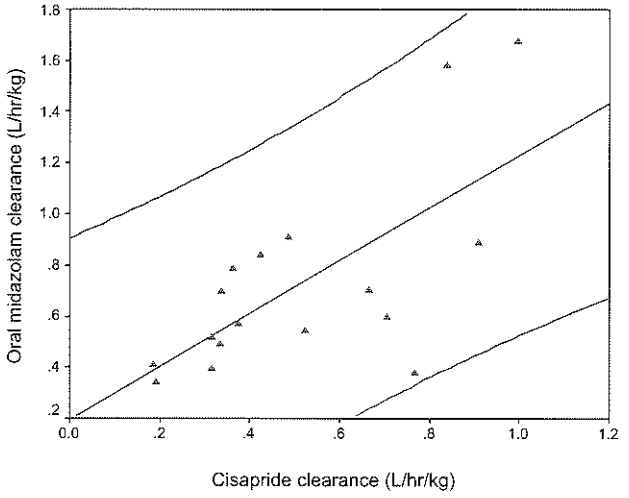

Figure 6 *Panel a: linear correlation (with 95 % CI) between IV midazolam clearance and oral cisapride clearance (r^2 = 0.47, P = 0.01). Panel b: linear correlation (with 95 % CI) between the apparent clearance for oral midazolam and cisapride (r^2 = 0.46, P = 0.001).*

In addition to selectivity for CYP3A4, it is critical that any pharmacologic probe used to assess the activity of this enzyme *in vivo* not be transported by P-gp such as is the case

with erythromycin.[18,19] To this regard, our initial *in vitro* experiments revealed that cisapride does not appear to be appreciably transported by P-gp (Figure 1). In contrast, it appeared to be an inhibitor of P-gp activity as measured by vinblastine accumulation in a standard model system (i.e., L-MDR1 cells, Figure 3). Following the administration of therapeutic cisapride doses in adults, plasma concentrations fall approximately one order of magnitude below the apparent Ki[40] suggesting that inhibition may be unlikely at extra-intestinal sites where the transporter is located.[41] In contrast, P-gp inhibition may be significant at the local concentrations of cisapride reached in the lumen of the small intestine after oral dosing. While intestinal P-gp inhibition by cisapride may be irrelevant to its sole use as a pharmacologic probe, the incorporation of cisapride into multi-drug "cocktails"[42] may affect activity determinations for concurrently administered substances that are transported by P-gp.

Collectively, these findings along with the selectivity of cisapride biotransformation for CYP3A4 would suggest that pharmacokinetic parameters which reflect cisapride elimination may serve as useful surrogates to assess total CYP3A4 activity *in vivo*. To this regard, we characterized the pharmacokinetics of midazolam administered by the oral and intravenous route and of cisapride in a group of 17 healthy adult volunteers who did not have a history of ingesting xenobiotics (e.g., prescription drug products, natural products) known or suspected to alter CYP3A4 activity. The pharmacokinetic parameters for midazolam following intravenous and oral administration in our subject cohort (Table 1) were similar to those previously reported for the drug from multiple studies conducted in adults.[43-45] Also, the estimate of midazolam absolute bioavailability from mean AUC data in our subjects (i.e., 37 %) was in agreement with previously reported values from adults (36 %) and pediatric patients (35 %).[46] Similarly, the values for elimination T½ and Cl/F (Table 1) of cisapride following a single, small oral dose to our subjects were similar to those reported previously from adults.[40,47] The variability observed in the clearance estimates for both midazolam and cisapride were expected based upon the normal, wide intersubject variation reported for CYP3A4/5 activity in man.[14-16] Thus, the pharmacokinetic data obtained for both midazolam and cisapride in our adult subjects appeared to be "normal", thus permitting us to undertake a comparison that was focused on the examination of possible concordance between the disposition of the two drugs.

Our data examining the associations between cisapride and midazolam clearance demonstrate concordance between the disposition characteristics of these two CYP3A substrates in healthy adults. A significant, positive linear correlation was found between the $AUC_{0\rightarrow\infty}$ for intravenous and oral midazolam (Figure 5a). Concordance in this relationship was expected given the relative dependence upon hepatic and intestinal CYP3A4 for the metabolism of midazolam.[37] The fact that the intercept for this relationship was not zero was anticipated and is likely explained by organ-specific differences (e.g., liver *vs.* intestine) in the location and amount of CYP3A isoforms (i.e., both CYP3A4 and CYP3A5) capable of catalyzing the biotransformation of midazolam.[48]

Based upon an evaluation of correlation coefficients and the 95 % confidence limits for our regression analyses, the apparent association between the Cl/F for cisapride and the apparent plasma clearance for both intravenous (Figure 6a) and oral midazolam (Figure 6b) provides *in vivo* corroboration of the quantitative importance of CYP3A4 as a determinant of cisapride biotransformation and thus, its plasma clearance. Despite statistical significance found for these correlations, the resultant r^2 values would not suggest a high degree of predictability. Some of the discordance in these relationships likely reflects the relative contribution of the respective CYP3A isoforms (i.e., CYP3A4/5) and their location (e.g., intestinal vs. hepatic)[6] relative to the biotransformation of midazolam and cisapride. In light of the virtual specificity of cisapride as a CYP3A4 substrate [30-32] relative to midazolam,[39,48] some degree of discordance between the pharmacokinetics parameters (i.e., Cl/F and AUC) for cisapride and midazolam was expected. As well, inter-subject differences in the rate and/or extent of oral bioavailability of the two compounds within the subjects in our study cohort could have produced some of the variability observed in these relationships (Figures 6a and 6b).

Despite the variability observed in the pharmacokinetic relationships between cisapride and midazolam, concordance was apparent and suggests that an evaluation of cisapride disposition *in vivo* could provide a potentially useful tool to assess total CYP3A4 activity. Classically, the assessment of phenotype or activity for a given drug-metabolizing enzyme can be reflected by pharmacokinetic parameters that reflect elimination of the parent drug from the body (e.g., clearance, elimination rate constant) and *vide infere*, substrate turn-over. The accuracy of such pharmacokinetic approaches is directly related to the reliability associated with estimation of the parameter(s) of interest. As was done in

this study for midazolam, validated limited sampling algorithms[33,34] can be used to reliably estimate plasma drug clearance. A more simple and potentially attractive approach would be to use a single plasma concentration determination to predict the clearance of a pharmacologic substrate. This has been previously attempted by several groups for midazolam,[23,48,49] all of whom reported considerable variability with plasma midazolam and/or metabolite ratios from a single time point when used to estimate plasma clearance. In contrast, our data for cisapride demonstrated a significant and highly predictive (i.e., $r^2 = 0.96$) correlation between plasma drug concentration and $AUC_{0\rightarrow\infty}$ when a single 8 (Figure 7) or 12 hour post-dose plasma concentration was used. As well, measurement of a 12 hour post-dose plasma concentration ratio of cisapride:NORCIS was also highly correlated with the apparent plasma clearance of cisapride ($r^2 = 0.91$, P = 0.001). Accordingly, it would appear possible to reliably estimate cisapride plasma clearance, a surrogate measurement of total CYP3A4 activity, from a single eight or 12 hour post-dose plasma concentration of parent drug.

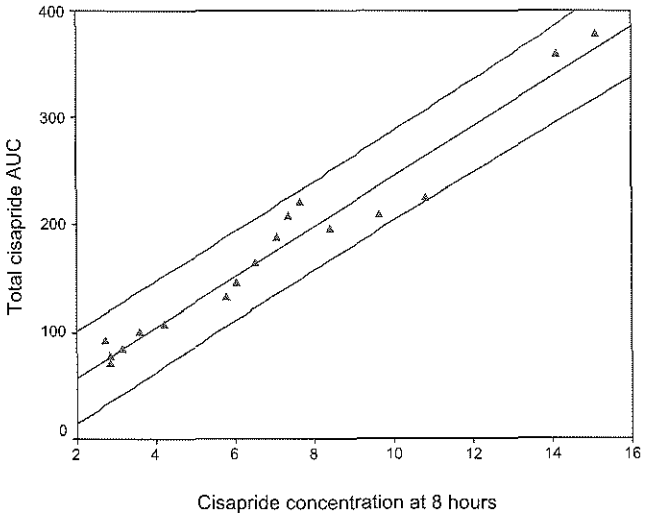

Figure 7 *Linear correlation (with 95 % CI) between cisapride AUC and cisapride plasma concentration at 8 hours ($r^2 = 0.96$, $P = 0.001$).*

When a single-point determination for prediction of drug plasma clearance is used, it is critical that the time points are selected based upon an understanding of the pharmacokinetic properties of the drug and/or metabolite of interest. In a study of midazolam, Lin et al.[23] chose a four hour time point based upon the average mean residence time (MRT) of the drug. Given that the MRT corresponds to the average time that the number of molecules absorbed reside in the body, choosing a time point earlier than the MRT may result in an underestimation of drug exposure and subsequent errant prediction of AUC and/or clearance. In the current study, the MRT of cisapride was 11.2 $\pm$ 4.1 hours. Thus, use of an 8 or 12 hour post-dose time to estimate AUC (or clearance) yielded a highly predictive relationship and hence, appears valid.

As denoted previously,[1] pharmacologic probe compounds used for phenotyping and/or the assessment of drug metabolizing enzyme activity should be well tolerated, have a minimal risk of producing adverse effects at the dose used and ideally, should accurately reflect enzyme activity at a no-effect dose. In this study, adverse effects associated with a single, low intravenous or oral dose of midazolam included drowsiness, ataxia, hiccups, diarrhea, vasovagal events, oxygen desaturation, and leg cramps. In contrast, no adverse events were noted in association with administration of cisapride. Despite the ability of therapeutic doses of cisapride administered chronically to increase the QTc interval and in rare instances, produce serious life-threatening cardiac arrhythmias,[24-28] the anticipated risks associated with the administration of a single, small oral dose of cisapride as a pharmacologic "probe" compound in subjects with a normal ECG and no other risk factors associated with prolongation of the QT_c interval would appear to be miniscule and acceptable. As well, the availability of a stable oral liquid formulation of cisapride and a documented safety profile in infants and children[24,25,28] could make this drug a suitable pharmacologic probe to evaluate CYP3A4 activity in pediatric patients, thus extending the range for experimental use.

10.9 Conclusions

Relative specificity for metabolism by CYP3A4, lack of transport by P-gp and apparent concordance between the dispositon of cisapride and midazolam supports the applicability of oral cisapride as a pharmacologic substrate to assess total CYP3A4 activity *in vivo*.

Cisapride plasma concentrations at 8 or 12 hours following a single subtherapeutic dose of the drug may prove useful as a single-point determination to reliably estimate cisapride plasma clearance and ultimately, CYP3A4 activity in humans. The investigational use of cisapride for this purpose appears warranted and should be further evaluated in both adult and pediatric populations to characterize the impact of ontogeny on total CYP3A4 activity (for pediatric patients) and to examine xenobiotic-enzyme interactions that are of potential clinical significance.

10.10 Acknowledgements

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Chapter 11

Single-Dose Pharmacokinetics of Cisapride in Neonates and Young Infants

Based on the article:
Single-Dose Pharmacokinetics of Cisapride in Neonates and Young Infants
Kearns GL, Robinson PK, Adcock KG, Wilson-Costello D, Knight GR, Zhou H-H, Wilson JT, Spielberg SP, Ward RE, van den Anker JN, and the Pediatric Pharmacology Research Unit Network.

11.1 Abstract

Cisapride, a prokinetic agent and substrate for CYP3A4, has been used to treat neonates and infants with feeding intolerance and apnea/bradycardia associated with gastroesophageal reflux (GER). Known developmental delay in the expression of CYP3A4 prompted us to conduct a classical pharmacokinetic (PK) study of cisapride in neonates and young infants. A total of 35 infants, 30 - 51 wk postconceptional age (PCA) at time of study, received a single oral cisapride dose (0.2 mg/kg) at 4 – 87 days postnatal age (PNA) followed by repeated (n = 6) blood sampling over 24 hr. Cisapride and norcisapride (NORCIS) were quantitated from plasma by HPLC-MS/MS and PK determined using non-compartmental methods.

Results

PK parameters (mean ± SD, (range)) available from a total of 32 subjects were: T_{max} – 4.4 ± 2.8 (1–12) hr; C_{max} – 29.3 ± 16.6 (5.1 – 71.7) ng/ml; elimination t½ – 10.7 ± 3.7 (1.9 – 18.1) hr; Cl/F – 0.62 ± 0.43 (0.2 – 1.9 l/hr/kg) and VD_{ss}/F – 9.0 ± 7.1 (2.9 – 30) l/kg. The apparent renal clearance (Cl_{ren}) of cisapride in infants (n = 28) with a complete urine collections was 0.003 ± 0.003 l/hr/kg. Sub-stratification of the population demonstrated a significantly lower Cl/F for cisapride (0.45 ± 0.26 l/hr/kg, $P < 0.05$) in the youngest infants (PCA 28 - 36 wk) as compared to infants with PCA of > 36 - 42 wk (Cl/F = 0.75 ± 0.46 l/hr/kg) and PCA of > 42 - 54 wk (Cl/F = 0.85 ± 0.69 l/hr/kg). A positive linear correlation was found between PCA and the apparent terminal elimination rate constant (λ_z) for cisapride ($P < 0.001$, $r^2 = 0.47$) but not with Cl/F. No association between PCA and the NORCIS:cisapride area under the curve (AUC) ratio was observed. All infants tolerated cisapride well without significant alteration in their QTc.

Conclusions

1. approximately 99 % of cisapride Cl/F in neonates and young infants was non-renal in nature;
2. Cl/F of cisapride in neonates and infants was reduced compared to data from older children and adults, likely as a result of developmental reductions in CYP3A4 activity;
3. as reflected by the correlation between PCA and λ_z, a rapid increase in total CYP3A4 activity occurs in the first 3 months of life and

4. lack of an apparent association between cisapride Cl/F and PCA may reflect variability in pre-systemic clearance from asynchronous developmental acquisition of hepatic and intestinal forms of CYP3A4.

11.2 Introduction

Cisapride is an orally administered prokinetic agent used for the treatment of gastroesophageal reflux disease and other gastrointestinal disorders (e.g., feeding intolerance, post-operative ileus, apnea/bradycardia associated with gastroesophageal reflux) in infants and chlidren.[1-3] Despite its association with the production of serious cardiac arrhythmias in patients with one or more specific risk factors for this rare adverse event,[4-5] more than a decade of clinical use and evidence from many controlled clinical trials conducted in both pediatric and adult patients suggests that the drug is safe and well tolerated.[1,2] Consequently, cisapride continues to be used in pediatric and adult patients throughout the world and in the United States, is available through a limited access protocol offered by the manufacturer (Janssen Pharmaceutical Research Foundation, Titusville, NJ) that is approved by the U.S. Food and Drug Administration.

An evolving concern with cisapride use in pediatrics focused in part upon its potential to alter cardiac conduction through its inherent effects on the IKr channel which is encoded by the human ether-a-go-go gene (HERG);[6] a particular concern in individuals with genetic polymorphisms in the HERG genes (LQT1 to LQT6) associated with production of the long QT syndrome.[7] Recent data from Yang, et al.[8] suggest that for specific mutations in gene coding regions for KvLQT1 and HERG (both of which encode pore channel forming proteins), the risk for drug-associated acquired long-QT syndrome (aLQTS) may be augmented. Experimentally, cisapride has been shown *in vivo* to produce an increase in the corrected QT (QTc) interval in healthy volunteers when combined with clarithromycin, an agent capable of producing minimal increases in QTc alone and in combination with cisapride, via a pharmacokinetic mechanism (i.e., inhibition of CYP3A4 with a subsequent increase in cisapride plasma concentration).[9] In a variety of recent clinical investigations performed in relatively small pediatric patient populations including neonates and young infants, cisapride-associated alterations in QTc have been examined with some studies reporting statistically significant increases[10-13] and

others [14,15] reporting no increases in QTc. Despite the association of cisapride treatment with QTc prolongation in neonates and infants, a single retrospective study (i.e., survey) examining data reported from over 58,000 premature infants, 19 % of who were treated with cisapride, reported that the apparent incidence of arrhythmias in premature neonates was less than 1/11,000.[16]

Another facet of the "concern" associated with cisapride use in premature infants resided with the belief that developmental immaturity of the enzymes responsible for cisapride metabolism produced accumulation of the drug and hence, increased the risk for concentration-dependent increases in QTc.[10,13] In humans, cisapride is extensively metabolized to norcisapride (NORCIS) via N-dealkylation (41-45 % of the administered dose) and to several minor metabolites.[17] Recent evidence has suggested that the N-dealkylation is primarily catalyzed by CYP3A4.[18-20] Pearce et al.[20] found that the biotransformation of cisapride to NORCIS occurred at concentrations that spanned the usual range observed with therapeutic administration of cisapride and that it was consistent with Michaelis-Menten kinetics for a single enzyme. Additionally, CYP3A4 catalyzed the formation of cisapride to NORCIS at rates greater than 100 times that found for non-CYP3A enzymes and more than 100- and 50-fold greater than that observed for CYP3A5 and CYP3A7, respectively. Thus, cisapride biotransformation to NORCIS is primarily catalyzed by CYP3A4 with other cytochromes P450 (including CYP3A5 and CYP3A7) not being quantitatively important. Finally, Treluyer, et al.[21] have recently shown that hepatic microsomes from fetuses and neonates less than seven days of postnatal age demonstrated little ability to catalyze the biotransformation of cisapride consequent to low CYP3A4 activity (in contrast to predominant CYP3A7 activity). Based on these data and other information that supports reduced CYP3A4 activity in the first month of life,[22,23] the potential for developmental differences in cisapride disposition is evident.

To assess the impact of ontogeny on cisapride disposition, we conducted a classical pharmacokinetic study of the drug in neonates (both term and preterm) and young infants. Our primary goals were to determine if developmental differences in CYP3A4 activity were apparent as reflected by cisapride elimination and also, if they were associated with surrogate measurements (e.g., postnatal and postconceptional age, body weight) that historically have been used to assess normal growth and development. A secondary study

goal was to examine the association between cisapride plasma concentrations and changes in the QTc interval following a single dose. The results from this study are reported herein.

11.3 Methods

Subjects

Thirty-six neonates and young infants were enrolled in this open-label, multi-center clinical investigation; 35 of which received the study medication. Subjects were eligible for enrollment if they met the following inclusion criteria:

1). regurgitation of oral feedings associated with one or more of the following symptoms:

a. an episode of apnea > 20 seconds in duration, or of any duration if accompanied by cyanosis, oxygen desaturation and/or bradycardia,
b. clinically significant bradycardia (< 100 beats/minute in neonates and < 80 beats/minute in infants > 1 month) and
c. increased gastric residual (> 2 ml/kg or 30 % of volume given) within three hours of a feeding;

2). body weight ≥ 800 grams;

3). an apparent medical need for prokinetic therapy as determined by an independent assessment performed by an attending Neonatologist not involved with the clinical trial; and

4). an informed consent (permission) document signed by the subject's parent(s) or legal guardian(s).

Subjects were excluded from participation if any of the following criteria were met:

a. currently active significant cardiovascular disease including ventricular arrhythmia, ischemic heart disease or heart failure;
b. current respiratory failure caused by nonpulmonary disease, or sinus node dysfunction;
c. history of prolonged QTc intervals, any cardiac conduction disturbance or *torsades de pointes*;
d. clinically significant vomiting, malnutrition or dehydration;
e. uncorrected electrolyte disorders such as hypokalemia or hypomagnesemia;

f. evidence of a chronic disease state as reflected by medical history, physical examination, and/or abnormal laboratory test results reflecting either clinically significant hepatic or renal compromise, hypoperfusion or the presence of anemia;
g. history of maternal drug or alcohol abuse within the past 12 months and/or a positive urine drug screen;
h. receipt of an investigational drug administered as part of a clinical trial within 30 days of study drug administration;
i. use (i.e., current or within 14 days of enrollment for both the subject and their mother if breastfeeding) of a prescription drug known to induce or inhibit cytochromes P450 and/or to prolong the QTc interval;
j. evidence of a condition and/or surgical procedure that could potentially interfere with gastrointestinal function and
k. inability to tolerate required study procedures (e.g., maintaining vascular access sufficient to enable repeated blood sampling).

All subjects were enrolled after informed parental consent (i.e., permission) was obtained. The study protocol was approved by the Institutional Review Boards (or Human Ethical Committees) of the participating clinical institutions (Children's Mercy Hospitals and Clinics, Kansas City, MO; Louisiana State University Medical Center, Shreveport, LA; Rainbow Babies and Children's Hospital, Cleveland, OH; Children's Hospital and Health Center, San Diego, CA; University of Utah, Salt Lake City, UT and the Sophia Children's Hospital, Rotterdam, the Netherlands). As well, the final study protocol was approved for conduct by the Pediatric Pharmacology Research Unit (PPRU) Network Steering Committee. A medical history, physical examination, and clinical laboratory tests (complete blood count, serum chemistry panel, liver function tests and urinalysis) were performed in each subject prior to enrollment and within 24 hours following administration of the first cisapride dose. As all subjects were confined to a hospital intensive care unit, they were continuously monitored (e.g., vital signs, ECG, oxygen saturation, general physical status) throughout the period of study.

Study Design and Drug Administration

This study was conducted as an open label, single-dose evaluation of cisapride pharmacokinetics at a single oral dose (0.2 mg/kg) using the proprietary formulation of the drug (Propulsid® Oral Suspension, 1 mg/ml, Janssen Pharmaceutical Research

Foundation, Titusville, NJ) derived from a single lot number. Each cisapride dose was prepared by a Pharmacist using a calibrated 2 ml oral dosing syringe designed to deliver the total dose. The study drug was administered by a Research Nurse who delivered the dose to the surface of the buccal mucosa or alternatively, placed the dose into an existing naso- or orogastic tube. Following cisapride administration, 5 ml of sterile water was administered to insure complete delivery of the cisapride dose to the gastrointestinal tract. All subjects were studied in an intensive care unit setting where they were continuously monitored by the Research Nurse and a qualified Neonatal Nurse and remained throughout the entire post-dose sample collection and observation period.

Sample Collection

Blood samples for determination of plasma concentrations of cisapride and norcisapride (NORCIS) were collected from an indwelling venous or arterial catheter placed to facilitate medical care unrelated to the pharmacokinetic study or in limited instances, from a properly performed heel puncture (i.e., capillary blood). Repeated blood samples (0.2 ml each) were obtained at the following times: pre-dose (time 0) and 0.5, 1, 2, 4, 8, 12 and 24 hours following cisapride dosing. Blood samples were initially placed into polypropylene tubes containing anticoagulant (0.9 mg K_2EDTA; Microtainer®, Becton Dickinson, Franklin Lakes, NJ), were immediately mixed by inversion and were centrifuged (2,500g for 10 minutes at 4°C) to separate plasma which was then removed by manual aspiration, transferred to polypropylene tubes and stored at -70°C within 30 minutes of collection. A six lead ECG tracing was obtained immediately after each of the aforementioned blood sampling periods to evaluate QTc intervals which were done "on site" by a qualified Pediatric Cardiologist and subsequently, by a Pediatric Cardiologist at a central reading facility. The initial ECG reading was performed as a safety assessment. The results from the latter (i.e., central) reading were inserted into the study database and used to assess relationships between cisapride plasma concentrations and QTc intervals. Vital signs were examined immediately prior to cisapride administration, hourly throughout the study and again with the post-study physical examination and laboratory safety assessment. Finally, in those infants who had an indwelling urinary bladder catheter placed upon medical indications that were not related to this study, a quantitative urine collection was performed for the 24-hour post-dose sampling period. For those infants without indwelling bladder catheters, urine was collected into wood pulp based study diapers (Tendercare®, RMED International, Inc., Delta, CO) that were placed at four-hour intervals and from which, urine was manually expressed after removal of the matrix

material to avoid fecal contamination. The total urine sample was mixed and the volume and pH of each urine sample were determined. An aliquot (10 ml) of the sample was then placed at -70°C where it was stored until determination of urinary cisapride concentrations.

Analytical Methods

Cisapride and NORCIS were quantitated from plasma (100 µl) and free cisapride from urine (500 µl) using a method validated by Janssen Research Foundation (Beerse, Belgium)[24] who kindly performed the analyses. Briefly, following a solid phase extraction, a 20 µl sample was injected onto the analytical column (C18 BDS-Hypersil, 500 x 4.6 mm, 3 µm, Alltech, Deerfield, IL). The analytes and internal standards (R187847 and R187846, Janssen Research Foundation, Berse, Belgium) were eluted along a gradient (25 % to 90 % organic methanol and 0.1M phosphate buffer) and detected by LC/MS/MS in positive ion-mode (API 3000 Triple Quadrupole Mass Spectrometer, Turboionspray™, MDS Sciex, Concord, Ontario, Canada). The range of linearity for this assay was 1 to 200 ng/ml for both cisapride and NORCIS, and the inter- and intra-assay coefficients of variation were consistently < 10 % for concentrations between 1 and 200 ng/ml. An eight point standard curve using the peak area ratio of active compound to internal standard was used to calculate all cisapride and NORCIS concentrations. The mean concentrations from the analysis of duplicate samples was reported and used to accomplish the pharmacokinetic and statistical analyses.

Pharmacokinetic Data Analysis

The maximal concentration (C_{max}) and time of C_{max} (T_{max}) for cisapride were determined for data from each subject by visual inspection of the plasma concentration versus time curve. The apparent terminal elimination rate constant (λ_z) was then estimated from linear, least-squares regression analysis of the plasma concentration vs. time profile for each analyte (i.e., cisapride and NORCIS). Area under the plasma concentration vs. time curve from time zero to the last sampling time point (AUC_{0-n}) for cisapride and NORCIS was calculated by using the mixed log-linear rule. The AUC was extrapolated to infinity ($AUC_{0\rightarrow\infty}$) by adding AUC_{0-n} to the area under the tail of each concentration vs. time profile calculated by the following equation: $AUC_{tlast\rightarrow\infty} = C_{tn} / \lambda_z$ where C_{tn} is the plasma concentration at the last sampling point predicted from the fit of the apparent terminal elimination phase. The apparent total plasma clearance (Cl/F) and steady state volume of

distribution (VD_{ss}/F) were calculated using standard non-compartmental techniques. Pharmacokinetic parameters for NORCIS were determined using a standard non-compartmental approach. In addition, the parent:metabolite (i.e., cisapride:NORCIS) plasma concentration ratios at each time point and also, for AUC_{0-n} were calculated and used as surrogate markers of CYP3A4 activity. Finally, the renal clearance (Clr) for cisapride was determined as follows: $Clr = Ae_{0-24hr} / AUC_{0-24hr}$ where Ae represents the cumulative amount of unchanged cisapride excreted in the urine over a 24 hours collection and AUC_{0-24hr} represents the corresponding plasma cisapride AUC for this period. All pharmacokinetic analyses were performed using the Kinetica software package (version 4.0, Innaphase, Inc, Philadelphia, PA). Results from the pharmacokinetic analyses are expressed as mean ± SD and 95 % confidence limits unless stated otherwise.

Statistical Analyses

Associations between selected pharmacokinetic parameters for cisapride and NORCIS and PCA were examined using both linear and nonlinear regression analysis with un-transformed data. Standard approaches to assess goodness-of-fit (e.g., r^2 values, sum of squared residuals) were used to evaluate all regressions. Cisapride pharmacokinetic parameter estimates between PCA subgroups were compared using the Wilk Shapiro test to assess homogeneity of variance, following by a two-tailed, unpaired Student's t-test with the Bonferroni correction applied for multiple comparisons as appropriate. Statistical analyses were performed using the SPSS software package (version 10.1, SPSS Inc., Chicago, Il) wherein a significance limit of $\alpha = 0.05$ was accepted.

11. 4 Results

A total of 35 infants (22 males, 21 Caucasians) ranging in postnatal age (PNA) from 4 to 102 days (41.2 ± 27.9 days), gestational age (GA) from 26 to 42 weeks (32.1 ± 5.9 weeks), PCA from 30 to 51 weeks (37.4 ± 5.1 weeks) and body weight from 1.0 to 6.6 kg (2.59 ± 1.26 kg) received the single oral dose of cisapride. One infant enrolled did not receive cisapride consequent to a change in their condition prior to dosing. A total of 25 infants were classified as preterm (GA ≤ 36 weeks at birth), 17 of which were male and 14 were Caucasian. Their mean (± SD) PNA, GA, body weight and length were 39.6 ±

24.4 days, 29.5 ± 2.8 weeks, 1.96 ± 0.61 kg and 42.9 ± 3.8 cm, respectively. Ten infants were classified as "full-term" (GA > 36 weeks at birth), five of which were male and seven were Caucasian. Their mean (±SD) PNA, GA, body weight and length were 45.3 ± 36.7 days, 38.7 ± 1.5 weeks, 4.13 ± 1.11 kg and 53.0 ± 4.1 cm, respectively. To assess the impact of development on the disposition of cisapride, the entire study population was subdivided into three groups based upon PCA (i.e., 28 to 36 weeks, > 36 to 42 weeks and > 42 to 54 weeks). The demographic data for these subpopulations are summarized in Table 1. As anticipated in a population of hospitalized neonates and infants, a total of 28 adverse events were reported in association with the single-dose study period; all of which were attributed to either pre- or co-existent medical conditions. However, the single oral dose of cisapride appeared to be well tolerated with no reported adverse events attributed to its administration.

Table 1 Demographic Characteristics of Study Population

Characteristics	**PCA 28-36wk**	**PCA > 36-42wk**	**PCA > 42-54wk**	**Total**
Total number (*n*)	**17**	**13**	**5**	**35**
Sex, *n* (%)				
Male	11(65)	7(54)	4(80)	22(63)
Female	6(35)	6(46)	1(20)	13(37)
Race, *n* (%)				
Caucasian	10(59)	9(69.2)	2(40)	21(60)
Non-Caucasian	7(41)	4(30.8)	3(60)	14(40)
PNA (days)				
Mean (± SE)	30.9(4.4)	40.8(8.6)	77.2(9.1)	41.2(4.7)
GA (weeks)				
Mean (± SE)	29.2(0.5)	33.2(1.4)	39.6(0.5)	32.1(0.8)
Weight (kg)				
Mean (± SE)	1.74(0.12)	2.96(0.25)	4.52(0.63)	2.59(0.21)
Length (cm)				
Mean (± SE)	41.7(0.8)	47.3(1.1)	56.4(0.8)	45.6(1.0)

Abbreviations include: PCA, postconceptional age; wk, weeks; PNA, postnatal age and GA, gestational age

The mean plasma concentration vs. time data for cisapride and NORCIS is illustrated by Figures 1 and 2, respectively. As anticipated, when the data for the entire study cohort were examined, there was considerable (i.e., approximately 14-fold) variability in the plasma concentrations for cisapride with apparent peak plasma concentrations (C_{max}) ranging from 5.2 to 71.7 ng/ml (mean ± SD = 29.3 ± 16.6 ng/ml). In contrast, the variability observed for the C_{max} of NORCIS was less, with concentrations that ranged from 1.1 to 9.3 ng/ml (3.41 ± 1.94 ng/ml). Variability was also noted for the time of peak plasma concentrations (T_{max}) for both cisapride (0.9 to 12.0 hours) and NORCIS (1.9 to 24.1 hours) with mean (± SD) / median values of 4.4 ± 2.8 / 4.0 hours and 8.2 ± 5.3 / 8.0 hours, respectively.

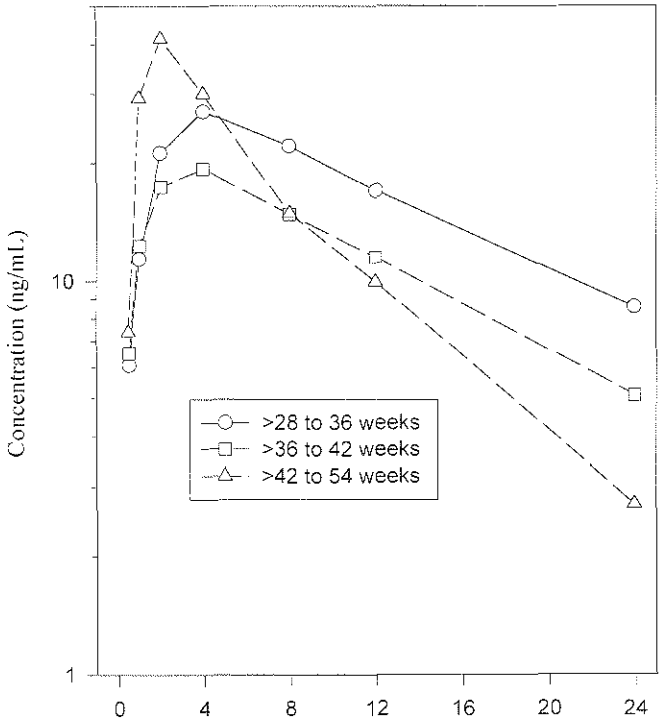

Figure 1 *Mean plasma concentration vs. time profile for cisapride following a single oral 0.2 mg/kg dose in subpopulations of neonates and infants stratified as a function of postconceptional age*

Examination of the mean plasma cisapride concentration versus time data for each of the PCA-based subgroups (Figure 1) illustrated trends suggestive of developmental dependence in drug disposition. Specifically, the apparent rate of absorption (i.e., reflected by a median value of 2.1 hours for T_{max}) was more rapid in the most mature infants (i.e., PCA category > 42 – 54 weeks). As well, the mean C_{max} was greatest for this group (mean value = 44.5 ng/ml) relative to the two younger and less mature groups of infants (i.e., PCA categories > 36 – 42 weeks and 28 – 36 weeks, mean C_{max} values = 23.4 and 30 ng/ml, respectively). Finally, examination of these data (Figure 1) revealed an apparent greater rate of decline for cisapride plasma concentrations in the more mature infants (i.e., PCA > 42 – 54 weeks) as compared to the two younger PCA subgroups.

The pharmacokinetic parameter estimates for cisapride determined in our study cohort are summarized in Table 2. Of the 35 subjects enrolled, 32 had sufficient plasma cisapride vs. time data to enable accurate estimation of two or more pharmacokinetic parameters. Intravenous access was lost in one of the subjects and hence, complete pharmacokinetic parameters could be reliably estimated for a total of 31 infants. Finally, a complete urine collection could not be accomplished in four infants and hence, renal clearance could be estimated for a total 28 infants.

As reflected by the pharmacokinetic data for cisapride (Table 2), the mean T_{max} for the most mature infants (i.e., PCA > 42 - 54 weeks, T_{max} = 2.2 hours) was significantly shorter than values observed for the infants in the 28 – 36 (T_{max} = 5.0 hours) or > 36 – 42 week (T_{max} = 4.4 hours) PCA groups. The same trend was found for the elimination half life (T½) with the mean value in the infants with a PCA > 42 – 54 weeks (4.8 hours) significantly lower than the values observed for the infants in the 28 – 36 and > 36 – 42 week PCA groups (i.e., 11.6 and 11.5 hours, respectively). The degree of systemic exposure to cisapride as reflected by the total area under the plasma concentration vs. time curve ($AUC_{infinity}$) was greater in the youngest, most immature infants (mean value = 568 ng/ml*hr, PCA 28 – 36 weeks) as compared to the values observed in the infants with PCA > 36 – 42 weeks (362 ng/ml*hr) and those with PCA > 42 – 54 weeks (364 ng/ml*hr). This specific difference was also reflected by an apparent plasma clearance (Cl/F) for cisapride that was lower in the infants with a PCA of 28 – 36 weeks (452 ml/hr/kg) as compared to the most mature infants (PCA >42 – 54 weeks, mean Cl/F = 846 ml/hr/kg). However, when cisapride Cl/F was compared between the neonates (i.e., PNA

≤ 4 weeks, $n = 15$) and infants (PNA range 32 – 87 days) in our study cohort, no statistically significant difference was found (0.49 ± 0.31 l/hr/kg for neonates *vs.* 0.71 ± 0.48 L/hr/kg for infants; P = 0.13). Finally, considerable variability was observed for both VD_{ss}/F and Cl/F across the three PCA-based patient subgroups, likely as the result of significant intersubject variability in the extent of cisapride bioavailability.

Finally, there was also considerable variability found for the estimates of renal clearance (Cl_r) for cisapride with no statistically significant differences in the parameter evident when the PCA-based subgroups were compared (Table 2). Using mean data for Cl/F (620 ml/hr/kg) and Cl_r (2.7 ml/hr/kg) for the entire study cohort, the approximate average non-renal clearance for cisapride (i.e., $Cl_{nr} = Cl/F - Cl_r$) was 617 ml/hr/kg. Thus, more than 99 % of Cl/F for cisapride was accounted for by non-renal routes (i.e., metabolism) for each of the three PCA-based patient subgroups.

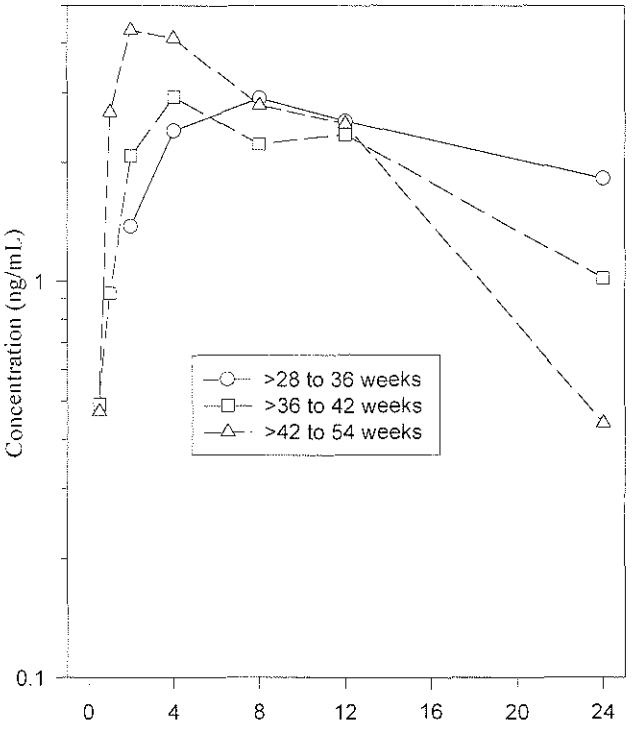

Figure 2 *Mean plasma concentration vs. time profile for norcisapride in subpopulations of neonates and infants stratified as a function of postconceptional age*

Table 2 Cisapride Pharmacokinetic Parameters

Parameters	PCA 28-36 weeks	PCA >36-42 weeks	PCA >42-54 weeks	Total
Total number of subjects	*17*	*13*	*5*	*35*
C_{max} (ng/ml)				
***n* =**	16	12	4	32
Mean (SD)	30.0 (17.5)	23.4 (11.7)	44.5 (19.6)	29.3 (16.6)
Min – Max	8.5 – 71.7	5.3 – 46.0	24.2 – 63.4	5.2 – 71.7
T_{max} (hours)				
***n* =**	16	12	4	32
Mean (SD)	5.0 (2.6)	4.4 (3.3)	2.2 (1.1)**	4.4 (2.8)
Median	4.0	4.0	2.1	4.0
Min – Max	2.0 – 12.0	1.0 – 12.0	1.0 – 3.7	0.9 – 12.0
$T_{1/2}$ (hours)				
***n* =**	15	12	4	31
Mean (SD)	11.6 (3.0)	11.5 (3.0)	4.8 (3.0)**	10.7 (3.7)
Min – Max	7.0 – 18.1	7.4 – 17.3	1.9 – 8.3	1.9 – 18.1
AUC_{last} (ng•h/mL)				
***n* =**	16	12	4	32
Mean (SD)	400 (184)	275 (149)	328 (209)	344 (179)
Min – Max	145 – 723	79 – 567	100 – 607	80 – 723
$AUC_{infinity}$ (ng•h/ml)				
***n* =**	15	12	4	31
Mean (SD)	568 (257)**	362 (199)	364 (249)	462 (250)
Min – Max	191 – 1031	117 – 776	107 – 706	107 – 1031
VD_{ss}/F (l/kg)				
***n* =**	15	12	4	31
Mean (SD)	7.4 (4.7)	12.7 (9.1)	4.1 (1.5)	9.0 (7.1)
Min – Max	3.2 – 17.3	3.8 – 30.5	2.2 – 5.6	2.2 – 30.5
Cl/F (ml/h/kg)				
***n* =**	15	12	4	31
Mean (SD)	452 (264)**	754 (462)	846 (697)**	620 (433)
Min – Max	194 – 1049	258 – 1715	283 – 1864	194 – 1864
Clr (ml/h/kg)				
***n* =**	13	12	3	28
Mean (SD)	2.9 (2.8)	2.8 (3.6)	1.5 (1.6)	2.7 (3.0)
Min – Max	0.16 – 8.5	0.14 – 12.0	0.38 – 3.3	0.14 – 12.0

Abbreviations include: PCA, postconceptional age; C_{max}, apparent peak plasma concentration; T_{max}, time of C_{max}, $T_{1/2}$, apparent terminal elimination half life, AUC_{last}, area under the curve from time 0 to last measurable plasma concentration; $AUC_{infinity}$, AUC extrapolated to infinity, VD_{ss}/F, apparent oral steady state volume of distribution; Cl/F, apparent oral total plasma clearance and Clr, apparent renal clearance
** denotes number of subjects with sufficient data to enable accurate estimation of parameter*
*** denotes $p < 0.02$ for comparison of parameter between respective PCA-based groups*

The pharmacokinetic parameter estimates for NORCIS are summarized in Table 3. As with cisapride (Table 2), considerable intersubject variability was seen for each of the pharmacokinetic parameter estimates. The apparent mean C_{max} for NORCIS in the most mature infants (i.e., PCA > 42 – 54 weeks, C_{max} = 5.04 ng/ml) was approximately 57% higher than observed for the younger infants (PCA groups 28 – 36 and > 36 to 42 weeks; mean C_{max} = 3.2 ng/ml) and upon visual inspection of the plasma concentration vs. time curve (Figure 2), appeared to decline more rapidly. As illustrated by Figure 2, the mean T_{max} for NORCIS (8.2 hours) was substantially longer than that for cisapride (4.4 hours), a finding consistent with the expected delay associated with the biotransformation of cisapride to its major metabolite. As well, the apparent rate of NORCIS elimination reflected by the mean plasma concentration data for infants in the > 28 to 36 week PCA group appeared to be more prolonged (i.e., slower) in comparison to the infants with PCA > 36 weeks. However, unlike the data for cisapride, no statistically significant differences were evident for the NORCIS parameters when compared between the patient subgroups. Our inability to administer NORCIS as a pure compound precluded accurate estimation of the plasma clearance, elimination T½, VDss and total AUC ($AUC_{infinity}$) for this metabolite as measurable concentrations of the parent drug were present throughout the 24 hour post-dose sampling interval (Figure 1). However, examination of the NORCIS:cisapride ratio for the AUC from time zero to the last measurable plasma concentration (i.e., AUC_{last}) suggested that the approximate extent of conversion of cisapride to NORCIS following a single 0.2 mg/kg oral dose was 17%, with a range of values from 4 to >100% that reflect an approximate 25-fold variability.

The apparent differences evident for cisapride disposition as a function of PCA (Table 2) prompted us to evaluate our data for potential correlations between PCA and selected pharmacokinetic parameters. As illustrated by Figure 3, a statistically significant, positive linear correlation (P = 0.001, r^2 = 0.69) was found between PCA and the apparent terminal elimination rate constant for cisapride. A similar but albeit less predictive (i.e., r^2 = 0.42) association was found between PCA and the cisapride elimination T½ (data not shown). When Cl/F, VDss/F and cisapride AUC were examined as a function of PCA, significant linear or nonlinear associations were not observed. Also, when the NORCIS:cisapride AUC ratio was examined as a function of PCA, no statistically significant association was found (Figure 4).

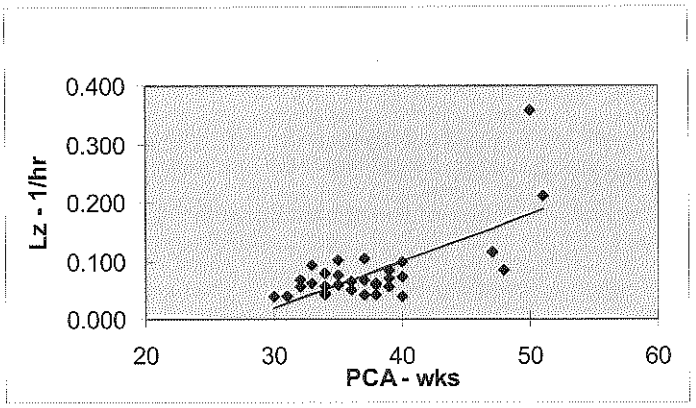

Figure 3 *Linear correlation between postconceptional age (PCA) and apparent terminal elimination rate constant for cisapride (Lz) in neonates and infants ($r^2 = 0.68$, $p < 0.001$)*

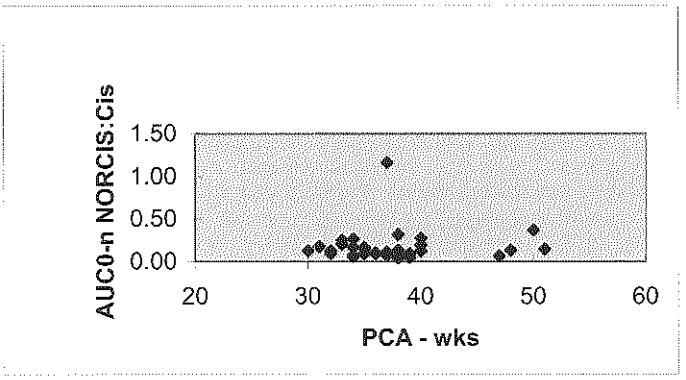

Figure 4 *Lack of an association between postconceptional age (PCA) and the plasma area under the curve (AUC_{0-n}) ratio for norcisapride (NORCIS) : cisapride (CIS)*

In addition to examining the disposition of cisapride, we performed an exploratory pharmacodynamic analysis to assess whether a relationship was evident between cisapride plasma concentration and cardiac conduction as reflected by the corrected (i.e., Bazette correction) QT interval. Figure 5 represents a plot of the cisapride plasma concentration as a function of the percent change in QTc interval (from coincident measurement) following the single oral 0.2 mg/kg dose. While the number of QTc observations with a change (from baseline) in a positive direction exceeded those in a negative direction, no association with the plasma cisapride concentration was evident. As well, the QTc values

observed in our study cohort were of similar magnitude to those reported previously from a controlled clinical trial neonates and young infants, irrespective of cisapride administration.[11]

11.5 Discussion

As stated previously, the wide use of cisapride in the medical treatment of neonates[16] and infants[1-3] and concerns regarding concentration-related prolongation of QTc interval (and predisposition for serious cardiac arrythmias) in neonates and infants receiving the drug[2,3,10-13] produced a need to study the clinical pharmacology of this drug in neonates and young infants. While data from a population pharmacokinetic study of cisapride in infants with gastroesophageal reflux[25] and plasma concentration data from an investigation of the drug in young infants[11] have been previously reported, there was no published information that described the disposition of cisapride and NORCIS in neonates; the pediatric population theoretically most susceptible to experience accumulation of cisapride in plasma consequent to developmental reductions in CYP3A4 activity.[26] Hence, the primary objective of the present study was to fill this information gap using a classical pharmacokinetic approach sufficient to enable critical examination of the impact of ontogeny on both cisapride disposition and metabolism.

In our study cohort, plasma cisapride concentration *vs.* time data sufficient to accurately characterize the apparent elimination rate constant and therefore, determine all pharmacokinetic parameters were available in 31 of 35 subjects (i.e., 88.6 % of subjects completely evaluable). Selected pharmacokinetic parameter estimates for our entire study cohort (e.g., mean T½ = 10.7 hr, Cl/F = 0.62 l/hr/kg; Table 2) were in agreement with those (i.e., T½ = 11.5 hr, Cl/F = 0.54 l/hr/kg) previously reported from a population pharmacokinetic study of 49 subjects (GA 24 – 42 weeks, birth weight 516 – 4770 grams, PNA 14 – 199 days) who received oral cisapride doses ranging from 0.11 to 0.45 mg/kg (mean dose = 0.18 mg/kg).[25] These respective estimates of cisapride T½ are considerably greater than the population average for this parameter reported from adult subjects,[27] a finding suggestive of developmental differences in its elimination. While the examination of the relationship between uncorrected cisapride Cl/F (i.e., expressed as l/hr) and body weight conducted by Preechagoon et al.[25] suggested that the degree of maturation in neonates and infants was influenced by development (i.e., a dependency of Cl/F on

weight), no association with subject age was presented. As well, these authors also found that the apparent volume of distribution for cisapride was not influenced significantly by either PNA or body weight.

Somewhat in contrast to these previous findings, our study demonstrated clear age-dependence for the disposition of cisapride in neonates and young infants. As reflected by the data in Table 2, the extent of cisapride systemic exposure (reflected by AUC) following a single 0.2 mg/kg oral dose was greater in the youngest, most immature infants (i.e., PCA 28 – 36 weeks); a finding associated with a lower value for Cl/F in this age subgroup (i.e., mean value - 0.452 l/hr/kg). The mean Cl/F in the most mature infants (i.e., 0.846 L/hr/kg) was almost double the value observed in the 28 – 36 week PCA group and was associated with a significant reduction in the mean elimination T½ (i.e., 4.8 hours vs. 11.6 hours). Examination of the correlation between the terminal elimination rate constant for cisapride and PCA (Figure 3) reflected significant predictability in the relationship (i.e., $r^2 = 0.68$) and most importantly, that the average value for λ_z increases approximately 4-fold for infants ranging in PCA from 30 to 50 weeks. As in the one previous pharmacokinetic study of cisapride in neonates,[25] we did not find any apparent developmental association for the VD_{ss}/F of cisapride. Finally, our finding of a significantly reduced mean Tmax (i.e., 2.2 hours) in the infants with a PCA of > 42 – 54 weeks as compared to the values for this parameter in the two other PCA-based subgroups (i.e., 5.0 and 4.4 hours for PCA 28 – 36 weeks and > 36 - 42 weeks, respectively; Table 2) is likely associated with developmental differences in gastrointestinal motility that increase the rate at which orally administered drugs are presented to the small intestine (i.e., site of absorption).[26]

Given that cisapride biotransformation is virtually dependent upon CYP3A4 activity [18-20] and that reduced CYP3A4 activity occurs in neonates and young infants,[21,22] our pharmacokinetic findings were expected. As previously shown by Lacroix, et al.[22] using human liver microsomes prepared from neonates and young infants, CYP3A4 activity (reflected by testosterone 6β hydroxylase activity) increased by approximately 3.3-fold during the first three months of life. This same dramatic increase was reflected by the association between PCA and the cisapride λ_z in our subjects (Figure 3); a pharmacokinetic parameter that reflects drug elimination (i.e., clearance of the parent molecule) and is not impacted (as determined) by factors that contribute to

bioavailability.[26] This particular finding is also corroborated by previous pharmacokinetic studies of CYP3A substrates conducted in neonates and infants as recently summarized by de Wildt, et al.[28] Specifically, previous studies using intravenous midazolam, a pharmacologic probe to assess CYP3A4/5 activity,[29] have demonstrated reduced plasma clearance (as compared to children and adults)[30,31] and dramatic, age-dependent increases in the activity of CYP3A as reflected by an increase in midazolam clearance from 1.2 ml/min/kg (in infants with GA < 39 weeks) to approximately 9 ml/min/kg in infants greater than 3 months of age.[32] In view of recent *in vivo* data that demonstrates the utility and selectivity of oral cisapride as a pharmacologic probe to assess total (i.e., hepatic and intestinal) CYP3A4 activity[33] and the relative non-selectivity of midazolam to assess CYP3A4 activity (i.e., its metabolism by CYP3A5),[29,34] our pharmacokinetic data illustrating the dependence of development on cisapride elimination (Figure 3) provides an accurate depiction of the impact of ontogeny on CYP3A4 activity during the first three months of life.

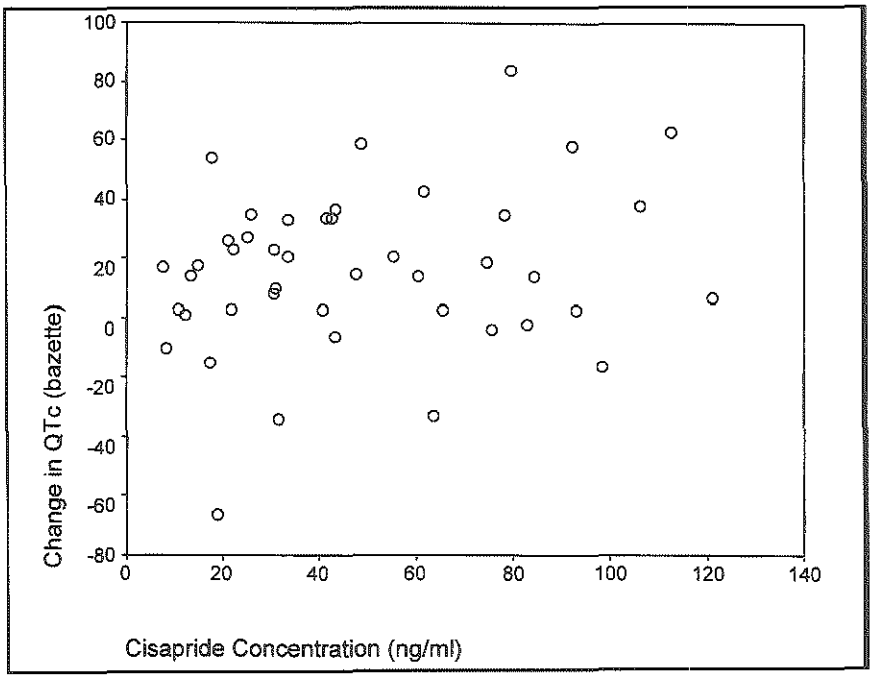

Figure 5 *Lack of an association between the post-dose cisapride plasma concentration and the change in QTc interval from baseline (i.e., pre-dose).*

As noted above, we elected to use PCA (as opposed to PNA or GA) to examine the potential for developmental dependence in cisapride disposition. Clearly, both PNA and GA provide surrogate "measurements" to assess the impact of development; the former reflecting post-birth events (i.e., *extra-utero* adaptation/influences) and the latter, organ development/function more reflective of *in utero* maturation at the time of birth. Postconceptional age (PCA = PNA + GA) serves to provide a hybrid surrogate for development, capturing the impact of maturation on organ size/function and body composition influenced by both *in-utero* and *extra-utero* events. The utility of using PCA to examine developmental dependence on pharmacokinetics has been shown for both renally excreted compounds (e.g., gentamicin)[35] and drugs that are extensively metabolized (e.g., morphine).[36] As well, the *in vitro* data of Lacroix, et al.[22] which includes assessment of fetal, neonatal and infant expression suggests that the impact of development on CYP3A4 activity is best reflected by consideration of both *in-utero* and *extra-utero* events.

As we[20] and others[17-19,21] have shown, the biotransformation of cisapride to NORCIS in humans is extensively catalyzed by CYP3A4, reflected in part by the extremely low Cl_r which we observed in our study cohort (Table 2). Also, in a recent study conducted in healthy adults, we demonstrated concordance between the AUC ratio of NORCIS:cisapride (as a surrogate to evaluate CYP3A4 activity) and the plasma clearance of midazolam.[33] Collectively, these findings prompted us to evaluate the AUC ratio of NORCIS:cisapride with regard to its potential to exhibit developmental dependence and thereby, reflect the impact of ontogeny on CYP3A4 activity.

As depicted in Figure 4, we did not observe a significant association between PCA and the NORCIS:cisapride AUC ratio, nor did we observe statistically significant differences for the NORCIS pharmacokinetic parameters between the three PCA-based patient subgroups (Table 3). This is not to say that the pharmacokinetics of NORCIS *per se* as they relate to cisapride do not mirror CYP3A4 activity but rather, that potential limitations of our study and developmental "confounders" precluded our ability to observe differences in CYP3A4 activity reflected by the metabolite data. Specifically, the observed plasma concentrations of NORCIS following a single cisapride dose were quite low (i.e., C_{max} range – 1.1 to 9.3 ng/ml; Table 3) and throughout the 24-hour post-dose sampling period, quantifiable plasma concentrations of cisapride were present.

Table 3 Norcisapride Pharmacokinetic Parameters

Parameters	PCA 28-36 weeks	PCA >36-42 weeks	PCA >42-54 weeks	Total
*Total number of subjects**	*16*	*12*	*4*	*32*
C_{max} (ng/ml)				
Mean (SD)	3.2 (1.0)	3.2 (2.7)	5.0 (1.7)	3.4 (1.9)
Min - Max	1.9 – 4.8	1.1 – 9.3	2.7 – 6.6	1.1 – 9.3
T_{max} (hours)				
Mean (SD)	8.9 (5.1)	8.2 (5.6)	5.7 (4.4)	8.2 (5.3)
Min - Max	2.0 – 24.1	2.3 – 24.0	1.9 – 12.2	1.9 – 24.1
AUC_{last} (ng•h/ml)				
Mean (SD)	51.7 (20.0)	46.1 (48.7)	46.6 (25.7)	48.9 (33.3)
Min – Max	19.0 – 86.0	6.8 – 148.9	20.9 – 81.8	6.8 – 148.9
Norcisapride:Cisapride AUC_{last}				
Mean (SD)	0.15 (0.06)	0.20 (0.31)	0.18 (0.13)	0.17 (0.19)
Min - Max	0.06 – 0.26	0.04 – 1.16	0.07 – 0.37	0.04 – 1.16

Abbreviations include: PCA, postconceptional age; C_{max}, apparent peak plasma concentration; Tmax, time of C_{max}, AUC_{last}, area under the curve from time 0 to last measurable plasma concentration

** denotes number of subjects with sufficient data to enable accurate estimation of parameter*

Collectively, this limited our ability to precisely determine (i.e., in the absence of parent drug) the λ_z for the metabolite which is required to accurately calculate $AUC_{inifinity}$ to determine apparent plasma clearance. As well, recent data from Leeder, et al.[37] examining the effect of age on the fractional recovery of 3-hydroxymorphinan (a CYP3A4 catalyzed metabolite of dextromethorphan) demonstrated a dramatic increase in activity during the first three to four months of postnatal life, presumably associated with the impact of ontogeny on the expression of CYP3A4 activity in the small intestine. Thus, given that cisapride was administered orally to our subjects who, by virtue of known patterns of developmental expression[37] would be expected to have demonstrable CYP3A4 activity in their enterocytes, intestinal conversion of cisapride to NORCIS may have been incomplete. This, in turn, would be expected to introduce considerable variability in the AUC ratio for NORCIS:cisapride when limited in calculation to the post-dose sampling interval (i.e., AUC_{0-n}). This was indeed observed in our study cohort as reflected by an approximate 25-fold difference in this ratio (i.e., 0.04 – 1.16; Table 3). As well, intestinal biotransformation of cisapride would be expected to markedly influence the extent of bioavailability, thereby producing wide variability in the calculated parameters VD_{ss}/F and Cl/F (Table 2) and potentially obscuring any association with PCA with a relatively

small study cohort. As a result, the apparent absence of correlations between PCA and either of these pharmacokinetic parameters was anticipated.

Despite our findings of developmental dependence in CYP3A4 activity reflected by cisapride pharmacokinetics, our data shed some light regarding the extent of "normal" interindividual variability associated with the expression of this isoenzyme during the early period of human development. As reflected by previously summarized data,[30] total CYP3A activity in healthy humans not exposed to xenobiotics capable of inducing or inhibiting the enzyme may vary from 10 to 20-fold. In our study cohort of neonates and young infants, variability in the AUC_{0-n} ratio of NORCIS:cisapride ranged from approximately 4-fold (in the PCA 28 – 36 week age group) to approximately 25-fold in the entire population (Table 3). While the lower degree of apparent variability in this ratio in the youngest, most immature infants might suggest that age-associated low CYP3A4 expression very early in life dampens the "normal" constitutive variability associated with this isoform, the limitations associated with our study (e.g., relatively short data collection interval, incomplete assessment of NORCIS pharmacokinetics) prevent corroboration of this assertion. Despite the aforementioned limitations in using this particular AUC ratio as a surrogate for CYP3A4 activity, this variability along with that observed for the other cisapride pharmacokinetic parameters that reflect elimination (e.g., T½, Cl/F; Table 2) suggests that in neonates and young infants, substantial variability in CYP3A4 activity exists in a magnitude similar to that observed for adults.

Finally, our exploratory pharmacodynamic analyses of ECG data provide some preliminary information as pertains to the purported concentration-effect relationship for cisapride to prolong the QTc interval in pediatric patients. In a recent study of 211 infants with suspected gastroesophageal reflux (84 of which were treated with cisapride and 127 of which were not), Benatar, et al.[11] reported an association between cisapride treatment and higher QTc intervals (as compared to controls). Cisapride plasma concentrations at steady state reported from this study ranged from 0 to 210 ng/ml (median peak/trough concentrations of 55.3/34.3 ng/ml); values that spanned those observed in our study cohort (Table 2). Despite an average QTc interval in the infants < 3 months of age who were treated with cisapride (i.e., 448.3 ± 25.5 msec) that was higher than observed in the control infants who were < 3 months of age (i.e., 419.5 ± 18.6 msec; $P < 0.001$), the range of QTc values observed in these two populations were similar and the authors reported no

correlation between QTc values and cisapride plasma concentrations. This is in agreement with our findings which demonstrated a lack of association between the post-dose, time dependent change in QTc interval and the corresponding cisapride plasma concentrations following a single oral dose (Figure 5).

11.6 Conclusions

The pharmacokinetics of cisapride in neonates and young infants are markedly influenced by development, especially those parameters that reflect elimination of the drug from the body. These developmental differences corroborate previous *in vivo* (i.e., pharmacokinetic) and *in vitro* data that support a clear, dynamic increase in CYP3A4 activity during the first three to four months of life. While not specifically assessed by this single-dose pharmacokinetic study, cisapride disposition in extremely young, immature infants (i.e., PCA 28 – 36 weeks) appears sufficiently different from older (more mature) infants to warrant age-based individualization of dosing regimens sufficient to prevent accumulation of the drug and the production of excessive systemic exposure. In contrast to previous studies of non-selective pharmacologic probe compounds used to assess total CYP3A activity (e.g., midazolam), the selectivity of cisapride for CYP3A4 provides, for the first time, an *in vivo* assessment of the ontogeny of this particular CYP3A isoform during the first three months of life. Finally, while cisapride can influence cardiac conduction by prolonging the QTc interval, preliminary pharmacokinetic-pharmacodynamic analyses do not suggest concentration or time dependent effects on QTc following administration of a single oral dose of the drug to neonates and young infants.

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Chapter 12

The Challenges of Delivering Pharmacogenomics into Clinical Pediatrics

Based on the article:
The Challenges of Delivering Pharmacogenomics into Clinical Pediatrics
Leeder JS and Kearns GL
Pharmacogenomics J 2:141-143 (2002)

12.1 Introduction

Progress in two major areas over the past 10 years has contributed to increasing optimism for improved understanding of pediatric disease and its pharmacologic management both now and in the future. First, a series of legislative changes targeted improved drug therapy in children of all ages. In 1994, the Food and Drug Administration (FDA) Pediatric Rule essentially provided an avenue for manufacturers to insert information pertaining to the clinical pharmacology and therapeutic use of drugs in pediatric patients into approved product labeling. The FDA Modernization Act (FDAMA) of 1997 provided an incentive for the pharmaceutical industry to complete pediatric studies of marketed drug products with patent life remaining while the FDA Final Rule of 1998 (effective April 1999) currently requires pharmaceutical companies to perform pediatric studies prior to filing of a new drug application (NDA) on new drugs that could benefit pediatric patients and/or are anticipated to be used in a substantial number (i.e., > 50,000) of pediatric patients. These legislative changes produced a dramatic increase in the number (i.e., > 400) of pediatric clinical trials conducted in the U.S.(mostly pharmacokinetic) involving more than 50,000 children ranging in age from birth to adolescence and resulted in expanded pediatric labeling for more than 30 drug products.[1]

The Human Genome Project, initiated in 1990 with the goal of sequencing the entire complement of human genes by the year 2005, is the second development that is expected to impact the diagnosis and treatment of pediatric diseases. This three billion dollar public investment is expected to lead to decreased morbidity and mortality through the development of more effective strategies to diagnose, treat and prevent human disease. Society has every right to expect that adults and children will be able to benefit equally from the promise of the genome era. In pediatrics, the newly acquired information has considerable potential to improve our understanding of:

1. genetic determinants of age-specific disease processes ,
2. genetic and developmental factors that contribute to the increased susceptibility of children to certain drug-related adverse events and
3. genetic factors that influence interindividual variability in drug disposition and response across the entire pediatric age spectrum.

Nevertheless, there are several factors unique to pediatrics that must be addressed before pharmacogenetic and pharmacogenomic strategies can be fully implemented in the

context of pediatric disease. We will provide some perspectives on these issues as well as current ethical and methodological concerns that impact the translation of these strategies to pediatric clinical medicine.

12.2 Application of Pharmacogenetics and Pharmacogenomics in a Developmental Context

Up to the present time, medical genetics has focused on unusual cases of Mendelian disorders, birth defect syndromes and chromosomal anomalies, generally within the setting of a pediatric tertiary medical center. With renewed interest in pediatric drug development and drug therapy over the past five years, the role of heredity can be expected to extend beyond diagnosis and surveillance to optimization of disease treatment - the ultimate goal of pediatric pharmacogenetics and pharmacogenomics. Defining the term "pharmacogenomics" as the study of how interacting systems of genes determine drug responses[2] is particularly appealing to pediatric health and disease since the concept of many genes acting in concert captures the essence of the developmental processes that characterize maturation from the time of birth through adulthood while retaining a focus on the individual. In the context of drug therapy, the fetus is considered to be relatively deficient with respect to drug metabolizing enzyme activities and postnatally, the acquisition of functional activity appears to occur in isoform-specific patterns (reviewed in 3). Furthermore, it is not unreasonable to expect that receptor systems and associated signal transduction pathways also undergo maturational changes as cognitive, verbal and social skills are acquired. Therefore, pediatric pharmacogenetics must identify the sources and consequences of inter-individual variability throughout development as they impact the disposition (i.e., pharmacokinetics) and action (i.e., pharmacodynamics) of drugs.

The Challenge of Pediatric Disease

Applying pharmacogenomic data derived from studies conducted in adults or experimental systems of adult origin may have limited applicability to pediatric disease. Disease processes affecting newborn infants (e.g., patent ductus arteriosus) or diseases of childhood such as Kawasaki's disease have no close correlates in adults. Similarly, acute lymphoblastic anemia, Wilm's tumor and neuroblastoma are all encountered during childhood and rarely, if at all, in adults. Several diseases with complex etiologies such as asthma, autism, attention deficit and hyperactivity disorder, cystic fibrosis, juvenile

rheumatoid arthritis and type I diabetes mellitus have their origins during childhood and are associated with age-related differences with respect to drug delivery, dosing and therapeutic response as compared to adults. Finally, certain forms of idiosyncratic toxicity such as Reye's syndrome associated with aspirin use,[4] valproate hepatotoxicity[5] and lamotrigine-induced cutaneous events[6] occur predominantly or at a considerably higher frequency in children implying that some determinants of susceptibility have a developmental component.

Genomic DNA obtained at any age or developmental stage can be used to identify specific single nucleotide polymorphisms (SNPs) or haplotypes of genes that contribute to the genetic liability (or genotype relative risk) for developing a specific complex disease as well as genes potentially predictive of an individual's response to pharmacotherapy. However, patterns of gene expression and the nature of the gene interactions that contribute to the pathogenesis of pediatric diseases (and thereby serve as potential targets for pharmacologic intervention) may only be discernable at specific critical points in the developmental continuum. For example, autism is one of a group of pervasive developmental disorders with lifelong consequences for affected individuals and their parents. The etiology of the disorder is poorly understood. It is thought to have genetic and environmental components and as recently suggested, may be a disease of early fetal development (reviewed in 7). While any understanding of how interacting networks of genes are deranged in a complex disease clearly requires an understanding of the normal patterns of development, a major challenge for developmental disorders like autism will be to identify the critical period during development when deviations from normal patterns will be most apparent, thereby knowing where and when to look.

The Challenge of Relevant Developmental Model Systems

Technological advances for studying gene expression (e.g,. microarrays; proteomics tools such as two-dimensional electrophoresis coupled with mass spectrometry and isotope coded affinity tags, among others) will allow raw DNA sequence from the Human Genome Project ultimately to be converted to information related to the functional integration of biological systems. In particular, the new field of modular proteomics has considerable potential from a developmental perspective since it attempts to rationalize how proteins interact to form functional modules that then organize into network architectures of biological systems.[8] In this context, identification of appropriate tissues or

model systems relevant to the target developmental stage represents a major challenge for pediatric pharmacogenomic endeavors. While tumor cells are readily accessible and provide exciting new possibilities for diagnosis and treatment using expression array technology,[9] appropriate tissue sources for microarray or proteomic investigations of neural development, the ontogeny of specific receptor and signal transduction systems or the ontogeny of drug metabolizing enzymes and transporters present more of a challenge for several ethical and logistical reasons. For example, "fetal research" broadly refers to the use of fetal cells and tissues generally obtained from induced abortions for the purposes of studying various aspects of cell physiology and human development and as such, is a topic of considerable moral, ethical and political controversy.[10]

Similarly, gene expression and proteomic investigations of human postnatal development require access to tissues of sufficient viability to allow isolation of intact mRNA and protein. Transplant quality pediatric tissue is relatively rare and priority should be given to its use to save the life of a suitable recipient. Likewise, diseased organs/tissues removed from transplant recipients may not be suitable for investigational use in that they often reflect the effects of disease superimposed upon normal function. Tissue biopsies have been used for pharmacogenetic investigations in adults[11] but unless they are indicated for medical diagnosis or management, they are not easily defensible ethically in pediatric patients since greater than minimal risk is involved in obtaining them solely for research purposes. Continuous cell lines and primary cell cultures are potential alternatives but may not totally replicate and over time, may lose the specific developmental phenotype of interest. The use of "humanized" transgenic animals holds some promise but thorough validation will be essential to ascertain their relevance to human development. Finally, the design of experimental systems to characterize the long term consequences of pharmacologic intervention (e.g. possible long term effects of psychotropics on the developing brain, an important concern in behavioral pediatrics[12]) is a challenge that will require considerable creativity and resourcefulness to overcome.

Special Ethical and Scientific Considerations

The Ethical, Legal and Social Implications (ELSI) research program has been an integral component of the Human Genome Project since its inception. Ethical issues related to genetic testing in children have been addressed by several groups of practitioners

including medical geneticists,[13] nurses[14] and pediatricians.[15] The common consensus from this program is:

1. that the primary justification for genetic testing in children and adolescents should be timely medical benefit to the child,
2. genetic testing should be preceded by informed parental consent and assent of older (i.e., ≥ 7 years) children, and
3. that genetic testing for adult-onset conditions generally should be deferred until adulthood or until an adolescent has developed mature decision-making skills.

Ethical issues considered for participation of children in clinical trials are most relevant to pharmacogenetic and pharmacogenomic strategies that may be enacted to ultimately impact the treatment of diseases/conditions such as asthma, epilepsy, cancer, transplant rejection, autoimmune diseases and inflammatory bowel disease, among others. Furthermore, the challenges (and potential rewards) are greatest for those conditions where the etiologies are poorly understood and as a consequence, the basis for rational pharmacotherapy is least evident. Consider the treatment of developmental and behavioral disorders such as autism, ADHD and obsessive-compulsive disorders in children where recent efforts provide an example of how clinical needs and challenges can be identified and prioritized, and strategies designed to meet those needs. The increasing use of psychotropic medications in preschool-aged children,[16] despite the absence of regulatory approval (i.e. approved pediatric labeling consequent to inadequate testing to demonstrate efficacy and/or safety in this specific age group), prompted the National Institute of Mental Health and FDA to convene a workshop with representation from clinicians and researchers, family and patient advocates, industry, ethicists and professional societies to specifically examine the need for psychopharmacologic intervention for young children.[17] The outcome of this workshop was several specific recommendations relating to the need for new, targeted treatment interventions based on improved understanding of the pathogenesis of various disorders and the need for studies of efficacy and safety of drugs used to treat those disorders in children at all developmental stages. These studies should include the ontogeny of receptors and drug metabolizing enzymes as well as the ontogeny of drug effects on the developing brain to elucidate the short- and long-term consequences of treatment.[9] While these needs present tasks that are somewhat daunting to the field of pediatric psychopharmacology, they are equally applicable to the treatment of other pediatric disorders. Since this information is necessary for proper treatment of children, the research is justified provided that special attention is paid to identifying and

minimizing potential risk, appropriate informed parental consent and child assent is obtained, specimens obtained for pharmacogenetic analysis (i.e., DNA) are used solely for the purposes described within a given research protocol and appropriate safeguards to protect the confidentiality of subject-derived information are enacted.[19,20]

12. 3 Summary and Conclusions

The post-genome area presents unprecedented opportunities to make significant contributions to clinical pediatrics through the purposeful and prudent incorporation of pharmacogenetic and/or pharmacogenomic "tools" into clinical trials designed to examine drug disposition, action, efficacy and potentially, the epidemiology of therapeutic drug use (*i.e.*, pharmacoepidemiology). Appropriate study design will be an increasingly important consideration as inevitable methodological difficulties are encountered.[20] For example, the number of children affected by a chronic disease such as arthritis is less than in adults and obtaining a sufficiently large study population to achieve statistical power represents a formidable challenge, especially when the frequency associated with a particular gene or allelic variant of interest is low. For diseases like asthma and ADHD where prevalence rates are much higher, the lack of objective diagnostic criteria poses a problem when establishing selection criteria for clinical trials and an obstacle for studies designed to establish roles for specific genes, SNPs or haplotypes in disease etiology or treatment outcome. For the revelations associated with this era of the human genome to be translated into discoveries with the potential to favorably impact the care and treatment of children, it will be incumbent upon society (i.e., both the scientific and lay public) to embrace the multiple opportunities afforded by new knowledge. This will require enhanced education of practitioners, parents and patients, and the development of new strategies that effectively integrate this "new science" into paradigms of clinical research and patient care. The greatest obstacle to achieving the potential of pharmacogenomics and pharmacogenetics to improve clinical pediatrics will likely not result from a lack of personnel and fiscal resources to meet the challenges but rather, treating a facet of science that is common to all pediatric patients as uncommon, unattainable and/or of limited clinical utility.

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Chapter 13

Omeprazole Disposition in Infants and Children: Role of Age and *CYP2C19* Genotype

Based on the article:

Omeprazole Disposition in Infants and Children: Role of Age and CYP2C19 Genotype

Kearns GL, Andersson T, James LP, Li BUK, Gaedigk A, Kraynak RA, Kuczmanski M, Ramabadran K, van den Anker JN and the PPRU Network.

13.1 Abstract

Omeprazole is frequently utilized to treat gastroesophageal reflux in infants and children despite the lack of age-specific pharmacokinetic and dosing information in the approved product labeling. To address this challenge, the potential influence of development and cytochrome P450 2C19 (*CYP2C19*) genotype on omeprazole disposition were examined by the conduct of two pharmacokinetic (PK) studies in children and adolescents (2-16 yr of age) after a single oral 10 or 20 mg dose of the drug. Plasma omeprazole concentrations were determined by HPLC-MS from 7 plasma samples obtained over a 6 hr post-dose period. Pharmacokinetic parameters were determined by non-compartmental methods. Subjects were genotyped for *CYP2C19* by PCR-RFLP.

Results

Data were available from 37 patients (19 female), 10 of which were ≤ 5 yr of age. No drug associated adverse events were observed. The numbers of functional *CYP2C19* alleles per subject in the cohort were: 2 ($n = 25$), 1 ($n = 11$) and 0 ($n = 1$). Pharmacokinetic parameters (mean ± SD, range) were: T_{max} (2.1 ± 1.2, 1-6 hr); C_{max} (331.1 ± 333.6, 20.8 - 885.8 ng/ml); $AUC_{0\rightarrow\infty}$ (809.5 ± 893.8, 236.9 - 1330.9 ng/ml*hr); t½ (0.98 ± 0.22, 0.7 - 1.4 hr); and Cl/F (1.8 ± 1.4, 0.3 - 5.8 l/hr/kg). Comparison of mean $AUC_{0\rightarrow\infty}$ values normalized for dose (i.e., per 1 mg/kg) between subjects with 1 vs. 2 functional *CYP2C19* alleles revealed no statistically significant difference. As well, the Cl/F and apparent elimination rate constant (λ_z) for omeprazole were not significantly different for subjects with 1 vs. 2 functional *CYP2C19* alleles. No association between age and either Cl/F, t½ or λ_z was observed. The range of t½ values for omeprazole were similar to those reported in adults (1-1.5 hr).

Conclusions

1. In children 2-16 yrs. receiving 10 or 20 mg of omeprazole as a single oral dose, the PK are quite comparable to values reported for adults and
2. in pediatric patients who are CYP2C19 extensive metabolizers, there was no association between genotype and the pharmacokinetics of omeprazole.

13.2 Introduction

Omeprazole, a substituted benzimidazole, has been shown to effectively suppress gastric acid secretion by inhibiting the H^+, K^+-ATPase (i.e., the proton pump) in the parietal cell. The degree of suppression is well correlated with systemic drug exposure reflected by the area under the plasma concentration vs. time curve (i.e., AUC) of the drug. Despite rapid elimination of omeprazole from plasma (i.e., mean elimination $t_{1/2} \approx 1$ hr), effect can persist for 24 to 72 hours consequent to strong binding of the active form to its target receptor.[1] Recent reviews chronicle information pertaining to its safety and clinical use in both adult[2] and pediatric[3] populations where the drug is used to treat a wide variety of disorders that benefit from modulation of intragastric pH.

Omeprazole is primarily metabolized in humans to hydroxyomeprazole by cytochromes P450 2C19 (CYP2C19), an enzyme that is polymorphically expressed, and to a minor extent by CYP3A, a polymorphically expressed enzyme that catalyzes the formation of omeprazole sulfone.[1] The affinity of omeprazole for CYP3A4 is approximately 10 times less than for CYP2C19 and as a result, it has the potential to produce drug-drug interactions only with other CYP2C19 substrates.[1] As well, the metabolism of omeprazole by both CYP2C19 and CYP3A4 appears to be stereoselective.[4]

Recent work confirms that the pharmacokinetics,[5,6] pharmacodynamics[7,8] and efficacy[9] of omeprazole is associated with and apparently, dependent upon CYP2C19 activity. The poor metabolizer phenotype for CYP2C19, produced by inheritance of two recessive loss-of-function alleles, is present in approximately 3 to 5% of Caucasians and African Americans and in approximately 15-20% of the Asian population.[10] While at least seven non-functional alleles have been identified to date (*CYP2C19*2-*8*),[11,12] the two most common variants (*CYP2C19*2-*3*) result from single nucleotide substitutions that convey dramatic reduction of enzyme activity.[12,13]

In a recent review, Zimmermann et al.[3] present data on the therapeutic use of omeprazole in infants, children and adolescents summarized from over 40 different clinical trials at daily doses ranging from 0.2 to 3.5 mg/kg/day. Hassall et al.[14] reported results from a multicenter study involving children 1 to 16 years of age treated with omeprazole for erosive reflux esophagitis. These investigators determined that the effective dose to

produce healing was between 0.7 to 3.5 mg/kg/day and that the weight adjusted (i.e., mg/kg) omeprazole dose in pediatric patients needed to be higher than required for adults consequent to purported age-associated differences in the pharmacokinetics of the drug. To date, three pharmacokinetic studies of omeprazole conducted in infants and children have failed to demonstrate a clear association between age and either apparent oral clearance or elimination half life.[15-17] None of these investigations examined the pharmacokinetic data as a function of *CYP2C19* genotype, a surrogate marker to assess phenotype.[18,19]

We report herein results from two independent investigations of omeprazole pharmacokinetics conducted in pediatric populations. These studies were designed to specifically examine the impact of development (i.e., age) and *CYP2C19* genotype on omeprazole pharmacokinetics, and to determine if a pharmacokinetic basis for age-specific dosing of omeprazole was indeed apparent.

13.3 Methods

Subjects

Children ($n = 37$, 2-16 years of age, both males and females) who had a therapeutic need for acid-modifying therapy as determined by their primary care physician but were otherwise healthy and within the 5% to 95% percentile for height and weight were eligible for enrollment in one of two pediatric pharmacokinetic studies of omeprazole:

1. a single-dose trial designed to characterize the pharmacokinetics of a 10 and 20 mg oral dose or
2. a single- and multiple-dose pharmacokinetic study of 10 and 20 mg oral doses.

Subjects were excluded from participation for any of the following: a recent history of alcohol or drug abuse, use of prescription or over-the-counter medications, evidence of any past or present abnormality in renal, hepatic, hematopoetic, gastrointestinal or central nervous system function, prior surgery on the gastrointestinal tract, any clinically significant abnormality found on a physical and/or biochemical examination conducted prior to omeprazole administration and participation in an investigational study within 30 days of enrollment. In addition, females who had attained menarche were eligible to participate only upon documentation of a negative urine pregnancy test. These studies

were approved by the Institutional Review Board at each of the participating clinical institutions and also, by the Network Steering Committee of the Pediatric Pharmacology Research Unit Network. All subjects were enrolled via informed parental consent and subject assent when appropriate (e.g., subjects ≥ 7 years of age).

Study Design and Observations

Both of the aforementioned studies were designed as open-label clinical trials to assess the impact of age and *CYP2C19* genotype on the disposition of omeprazole in pediatric patients. Subjects were required to discontinue prior treatment with omeprazole other proton pump inhibitors for 7 days and H_2-receptor antagonists for 24 hours prior to entering the study.

Following an eight-hour period of fasting, subjects were admitted to a clinical study facility on the morning of study where they remained for a period of approximately eight hours. Prior to administration of the study medication, an indwelling silastic venous cannula (22 GA, 1.0 inch, 0.9 x 25 mm BD Insyte® Autoguard®, Becton Dickinson Medical Systems, Sandy, UT) was inserted in the distal portion of an upper extremity to enable repeated blood sampling necessary to accomplish the pharmacokinetic objectives without repeated discomfort to the subjects. Patency of the cannula was maintained using a sterile solution of 0.9% sodium chloride for injection (Syrex, Neptune, NJ).

All subjects received either an intact omeprazole capsule of a single lot number or the contents of the capsule sprinkled on approximately one ounce (i.e., two tablespoonfuls) of applesauce (Mott's Applesauce, Williamson, NY). Subjects ≤ 20 kg received the 10 mg dose and those > 20 kg the 20 mg dose. All omeprazole doses were administered before breakfast with tap water (90 – 180 ml), followed by a two-hour period of additional fasting where only water (≤ 180 ml) was offered to subjects complaining of hunger or thirst. As well, subjects were asked to remain either recumbent or seated for the two-hour post-dose period and to refrain from exercise. After the initial two-hour period, subjects were provided with an age-appropriate meal and *ad libitum* access to water, clear fruit juices and non-carbonated, caffeine-free beverages. All study subjects received their dose of omeprazole between 0700 and 0900 hours so as to minimize any potential confounding effect of diurnal variation on drug disposition. A complete physical examination including

hematology, urinalysis and serum chemistry assessment were performed prior to study drug administration and again upon completion of the pharmacokinetic sampling period. Venous blood samples (2.0 ml each) were obtained from an indwelling cannula immediately prior to omeprazole administration (i.e., time = 0) and at 0.5, 1, 1.5, 2, 3, 4 and 6 hours post-dose. Samples were placed into glass tubes containing anticoagulant (sodium heparin; Vacutainer®, Becton Dickinson, Franklin Lakes, NJ), were immediately mixed by inversion and left at room temperature for 10 to 60 minutes until centrifugation (2,500g for 15 minutes). Plasma was then removed by manual aspiration and immediately placed at -20°C until analysis. Vital signs were examined periodically throughout the study and with the post-study physical examination and laboratory safety assessment. The study protocols were conducted under applicable Good Clinical Practice regulations in compliance with all regulatory requirements under the U.S. Food and Drug Administration IND # 19-810/S-074.

Data Analysis

Omeprazole was quantitated from plasma in duplicate using a validated HPLC method with tandem mass spectrometric detection.[20] The method had a lower limit of detection of 5.0 ng/ml and a range of linearity between 5 and 500 ng/ml. Inter- and intra-assay coefficients of variation for the method were consistently < 9% for concentrations throughout the range of linearity. Concentrations of the major metabolites of omeprazole (5'-hydroxyomeprazole, omeprazole sulfone) were not determined from the patient samples.

The cellular "waste" from pre- and post-dose anticoagulated blood samples required for pharmacokinetic analysis for each subject was shipped at 4°C and shipped to the Pediatric Pharmacology Research Unit Core Pharmacogenetic Laboratory (Kansas City, MO) within 10 days of collection. Genomic DNA was isolated from the cellular fraction of pooled blood specimens using the Qiagen blood kit (Qiagen, Chatsworth, CA). *CYP2C19* genotyping was conducted using procedures adapted from previous methods.[21,22] Additionally, assays for the detection of *CYP2C19*4* and **5* alleles were performed using similar PCR-RFLP techniques developed by one of the co-investigators (Dr. Andrea Gaedigk, unpublished method).

Pharmacokinetic analyses were conducted using standard non-compartmental approaches based upon Statistical Moment Theory. Individual peak plasma concentrations (C_{max}) and time of peak plasma concentrations (T_{max}) were determined by visual inspection of the plasma concentration versus time profile for each subject. The apparent terminal elimination rate constant for omeprazole (λ_z) was estimated from each subject by nonlinear regression analysis ($1/y^2$ weighting) of the omeprazole plasma concentrations measured between 3 and 6 hours. The area under the plasma concentration versus time curve from 0 to 6 hours post dose (AUC_{0-6}) was determined using the log-linear trapezoidal rule. Extrapolation of the AUC to infinity ($AUC_{0-\infty}$) was calculated by summation of $AUC_{0-Tlast} + Cp_{Tlast}/\lambda_z$, where Cp_{Tlast} represented the last quantifiable post-dose plasma concentration predicted from the fitted apparent terminal elimination phase. Apparent total body clearance (Cl/F) and steady state volume of distribution (Vd_{ss}/F) were calculated from the $AUC_{0\rightarrow\infty}$ using standard equations.

Examination of possible age dependence for omeprazole pharmacokinetic parameters was conducted using both linear and nonlinear regression analyses. Comparison of the omeprazole AUC (i.e., both AUC_{0-6hr} and $AUC_{0\rightarrow\infty}$) between respective *CYP2C19* genotypes was conducted using the Wilk-Shapiro test to assess homogeneity of variance for the pharmacokinetic parameters, followed by application of an unpaired, two-tailed Student's t test. All statistical analyses were conducted using Microsoft Excel® 2000 (Microsoft Corporation, Seattle, WA) and SAS version 6.0 (SAS Institute, Inc., Cary, NC). A significance limit of $\alpha = 0.05$ was accepted for all statistical analyses.

13.4 Results

Complete concentration vs. time profiles for omeprazole and *CYP2C19* genotype data were available from a total of 37 subjects (19 female 18 male; 26 Caucasian, 11 African American). The subjects ranged in age from 2 to 16 years (mean ± SD = 8.9 ± 3.7 yr), their height ranged from 31.3-68.5 inches (52.5 ± 8.8 inches) and their body weight ranged from 5.0 to 57.7 kg (25.6 ± 14.72 kg). The weight-adjusted dose of omeprazole ranged from 0.16 to 0.91 (0.41 ± 0.21) mg/kg across the two fixed-dose groups (i.e., 10 and 20 mg dose groups combined). All non-functional alleles were *CYP2C19*2*, no **3*, **4* or **5* alleles were detected. In the study cohort, a total of 25 subjects possessed two

functional alleles for *CYP2C19*, 11 subjects had one functional allele and one was a homozygous poor metabolizer (i.e., 0 functional alleles). All subjects tolerated the single dose of omeprazole and all study procedures well without apparent adverse events attributable to the study medication.

The considerable range (i.e., 5.7-fold) in weight normalized omeprazole doses in this study cohort produced, as expected, a wide variation in the plasma concentration vs. time profiles for the drug. Consequently, individual, mean or median plasma concentration *vs.* time data are not depicted graphically. Peak plasma concentrations were observed to occur between one and six hours, with a mean (± SD) T_{max} of 2.15 ± 1.21 hr. Data from 14 subjects were not included in the pharmacokinetic analysis reported herein given that either an apparent delay in omeprazole absorption (n = 3) or the unavailability of sufficient post-dose plasma concentrations consequent to loss of the indwelling intravenous cannula (n = 11) precluded accurate assessment of an apparent terminal elimination phase and hence, made reliable estimation of pharmacokinetic parameters impossible. Representative data from the single-dose pharmacokinetic study demonstrated high variability in the apparent peak plasma omeprazole concentration (i.e., C_{max}) with values ranging from 16.22 to 251.12 ng/ml (98.1 ± 87.6 ng/ml) and 43.41 to 1338.4 ng/ml (313.4 ± 343.6 ng/ml) for the 10 and 20 mg doses, respectively.

Figure 1 Omeprazole Terminal Elimination Rate Constant (λ_z) Expressed as a Function of Age

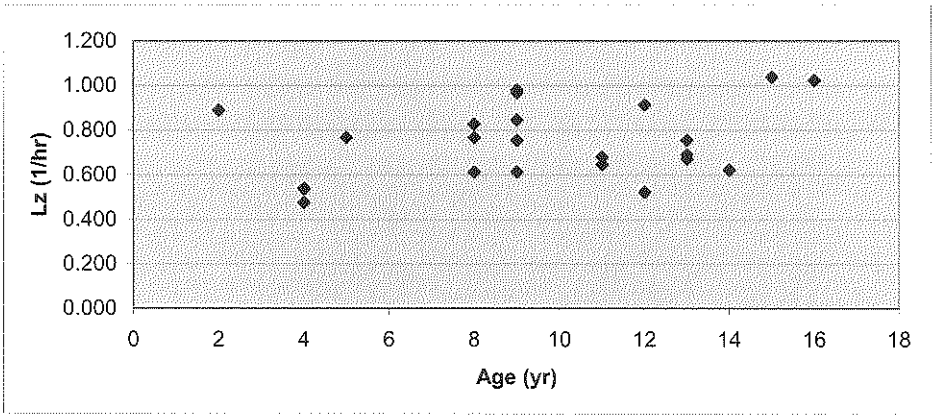

Figure 1 *Lack of an association between the apparent terminal elimination rate constant for omeprazole (Lz) and subject age.*

Table 1 Summary of Omeprazole Pharmacokinetic Data in Children and Adolescents ($n = 23$)

	$AUC_{0\rightarrow\infty}$ (ng/ml*hr)	C_{max} corr (ng/ml)	T_{max} (hr)	Cl/F (l/hr/kg)	VD_{ss}/F (l/kg)	T½ (hr)
Mean	809.50	446.21	2.15	1.76	2.60	0.98
S.D.	893.80	402.40	1.21	1.38	2.66	0.22
Median	624.49	218.14	2.0	1.59	2.14	1.04
Minimum	236.96	42.15	1.0	0.29	0.43	0.67
Maximum	1330.87	1449.46	6.0	5.80	12.16	1.45

Abbreviations include: $AUC_{0\rightarrow\infty}$ area under the plasma concentration vs. time curve extrapolated to infinity; C_{max} corr, apparent peak plasma concentration correct (i.e., normalized) to a 1 mg/kg dose; Tmax, apparent time of C_{max}; Cl/F, apparent oral plasma clearance; VD_{ss}/F, apparent volume of distribution; T½, apparent terminal elimination half life.
Data are from 23 subjects who had sufficient plasma concentration vs. time profiles to accurately characterize the apparent terminal elimination phase. All 23 subjects were CYP2C19 extensive metabolizers as characterized by the presence of either 1 or 2 functional CYP2C19 alleles by genotyping.

The pharmacokinetic data from 23 evaluable (i.e., complete plasma concentration vs. time profiles) subjects are summarized in Table 1. Considerable variability was noted for $AUC_{0\rightarrow\infty}$ (5.6-fold), Cl/F (20-fold), VD_{ss}/F (28.8-fold) and T_{max} (6-fold). Less variability was observed for the omeprazole terminal elimination rate constant (λ_z, 2.2-fold). Similar to a recent report of omeprazole steady state pharmacokinetics in children,[16] we did not find a statistically significant correlation between subject age and the dose-normalized (i.e., per 1 mg/kg) AUC for omeprazole ($r^2 = 0.03$, data not shown). As well, neither linear nor nonlinear associations were found between subject age and either VD_{ss}/F, Cl/F or λ_z, ($r^2 < 0.2$ and $p > 0.05$ for all correlations). The lack of an apparent developmental dependence for omeprazole disposition in the study cohort is illustrated by the data for λ_z (Figure 1), a pharmacokinetic parameter that is generally considered to be independent of omeprazole dose and/or extent of absorption as reflected by C_{max} when single doses of the drug have been administered to adults.[23]

Examination of omeprazole pharmacokinetic data as a function of *CYP2C19* genotype failed to yield statistically significant differences when either λ_z, or Cl/F were compared between the subjects with either one ($n = 11$) or two ($n = 12$) functional alleles. In contrast, the mean (± SD) uncorrected (i.e., for mg/kg dose) $AUC_{0\rightarrow\infty}$ for the subjects with one (820.44 ± 387.72 ng/ml*hr) functional *CYP2C19* allele was significantly ($P = 0.02$) greater than that observed for the subjects with two functional alleles (488.56 ±

298.97 ng/ml*hr). However, when the $AUC_{0\rightarrow\infty}$ values corrected for omeprazole dose (i.e., per 1 mg/kg) in subjects with one (1025.40 ± 822.27 ng/ml*hr) and two (929.36 ± 861.76 ng/ml*hr) functional *CYP2C19* alleles were compared, no statistically significant difference was apparent (Figure 2). Exploration of the apparent discrepancy between the assessment of $AUC_{0\rightarrow\infty}$ (i.e., un-normalized vs. weight-normalized) as a function of *CYP2C19* genotype revealed that by chance, the weight-adjusted dose of omeprazole received by the subjects with one functional allele (1.05 ± 0.55 mg/kg) was significantly ($p = 0.04$) greater than that received by the subjects with two functional alleles (0.63 ± 0.3 mg/kg).

Figure 2 Omeprazole AUC Corrected for a 1 mg/kg Oral Dose as a Function of *CYP2C19* Genotype

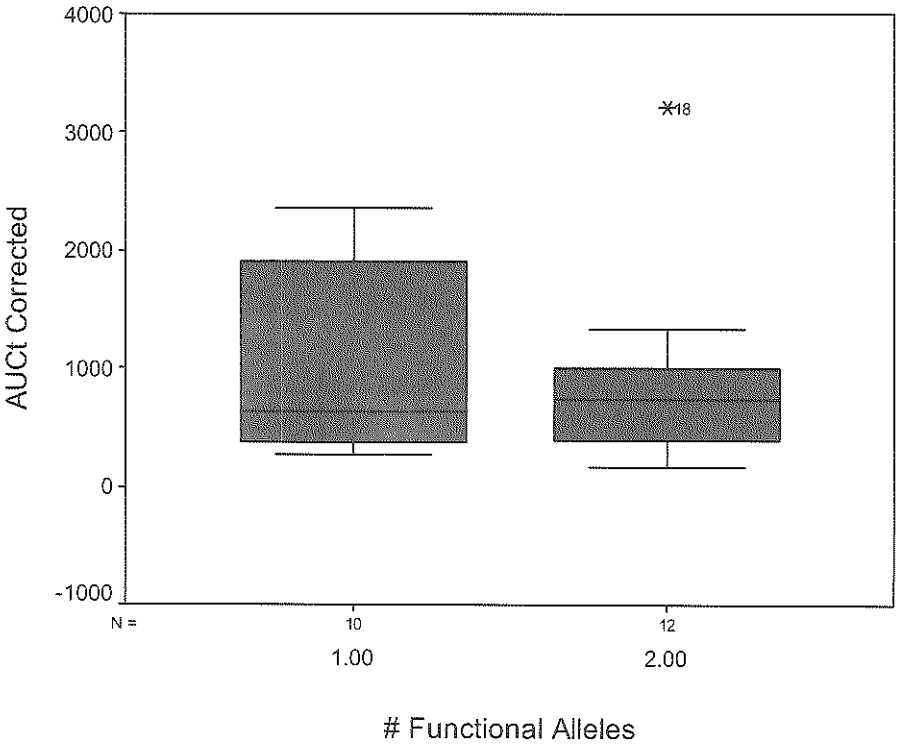

Figure 2 *Box plot of omeprazole area under the plasma concentration vs. time curve (AUC) normalized for omeprazole dose (i.e., per 1 mg/kg) comparing subjects with 1 (n=10) and 2 (n=12) functional alleles for CYP2C19. No statistically significant difference was evident when AUC was compared between subjects with 1 vs. 2 functional alleles. *18 denotes an outlier (subject #18) with 2 functional CYP2C19 alleles.*

Previous investigations and reviews[1,2,23,24] suggesting that the pharmacokinetics of omeprazole following single-dose administration were not concentration-dependent prompted us to examine the relationship between the weight-normalized dose of omeprazole and AUC for our study cohort. Significant variability in the values for both $AUC_{0\rightarrow\infty}$, $AUC_{0\rightarrow 6hr}$ and Cmax precluded demonstration of either a linear or nonlinear relationship (data not shown) with omeprazole dose (i.e., mg/kg). To further assess the potential for nonlinearity in omeprazole pharmacokinetics in our study cohort, the relationship between λ_z and the apparent peak plasma omeprazole concentration (C_{max}) was examined (Figure 3) and failed to yield a statistically significant association. Thus, linearity in the pharmacokinetics of omeprazole following a single 10 or 20 mg dose to pediatric patients is suggested.

Finally, only one subject in our study cohort was found to have no functional *CYP2C19* alleles and hence, was classified as a poor metabolizer. As expected, the AUC for omeprazole in this subject (i.e., 4551.4 ng/ml*hr) was considerably higher than the average values observed for the *CYP2C19* extensive metabolizers in our study cohort (Table 1).

Figure 3 Lack of an Association Between Omeprazole Cmax and Apparent Terminal Elimination Rate Constant (λz)

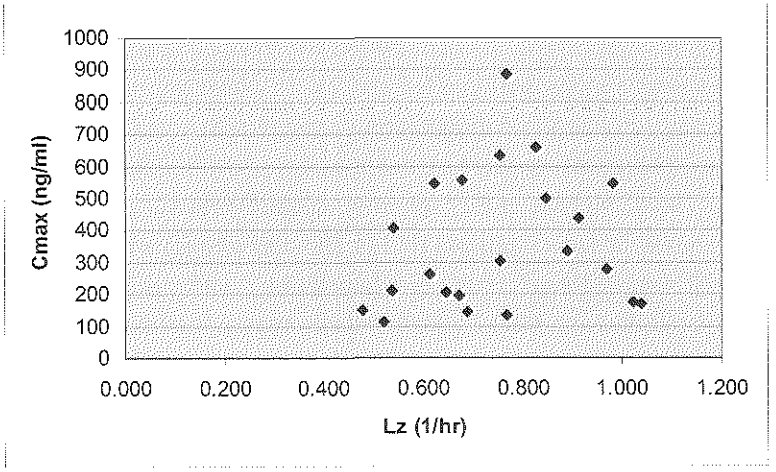

Figure 3 *Lack of an association between omeprazole apparent peak plasma concentration (C_{max}) and apparent terminal elimination rate constant (Lz).*

13.5 Discussion

Given that the pharmacodynamics and efficacy of omeprazole appear to be related to the degree of systemic exposure[2,3,7-9,24] and that the *CYP2C19* poor-metabolizer phenotype has been shown to be a major determinant of the pharmacokinetics (i.e., plasma clearance) and hence, the degree of systemic exposure from a given dose of the drug in adults,[1,5,6,23] there was clear reason and rationale for us to explore the association between *CYP2C19* activity (as reflected by genotype) and omeprazole disposition in pediatric patients. This assertion is supported by the current wide pediatric use of omeprazole and other proton-pump-inhibitors in treating acid-related gastrointestinal disorders (e.g., gastroesophageal reflux, duodenal and gastric ulcer disease, erosive esophagitis, gastritis, *H. pylori* infection) and the apparent wide range of omeprazole doses (i.e., 0.7 to 3.5 mg/kg/day) that are considered to be "therapeutic" in infants and children.[3,14] Despite a recent statement that "on a per-kilogram basis, the doses of omeprazole required to heal erosive esophagitis are much greater (in children) than those required for adults",[14] the currently used doses of this drug in pediatrics do not appear to be based upon age-associated differences in either its pharmacokinetics or pharmacodynamics but rather, on observed results from a variety of clinical trials and reports of use.[3]

To date, three publications[15-17] have reported pharmacokinetic data for omeprazole from pediatric patients. The data from these studies are summarized in Table 2. Similarities for all four pharmacokinetic studies reported are evident for the apparent elimination half life for omeprazole with mean values ranging between 0.9 and 1.6 hr which appeared to be independent of subject age and/or route of administration. Considerable variability was evident when omeprazole exposure was assessed by examination of AUC data for all of the aforementioned studies. Jacqz-Aigrain, et al.[15] reported values for $AUC_{0\rightarrow\infty}$ that ranged from 1.08 to 21.43 μg/ml*hr for 13 patients (0.3 to 20 years of age) who received a single intravenous dose (47.4 ± 27.2 mg/1.73m^2) of omeprazole. A subsequent study of omeprazole pharmacokinetics following intravenous administration to 9 infants and young children (0.4 - 2.3 years of age; dose = 0.9 ± 0.3 mg/kg) reported AUC values ranging from 0.55 to 7.71 μg/ml*hr. The mean ± SD total plasma clearance values between these two studies was dimensionally similar when those for younger infants[17] (0.53 ± 0.29 l/hr/kg) were compared to data from a population that included adolescents and young adults (0.36 ± 0.32 l/hr/kg).[15]

Table 2 Summary Pharmacokinetic Data for Omeprazole in Pediatric Patients

Study	N =	Age Range (yr)	Dose (mg/kg)	Route	Cl (l/h/kg)	$t^{1}/_{2\lambda z}$ (hr)
Jacqz-Aigrain E, et al.[15]	13	0.3-20.0	47.4 ± 27.2*	IV	0.36 ± 0.32	1.28 ± 0.88
Andersson T, et al.[16]	7	1.6-6.1	1.6 ± 1.0	PO	NR	0.89 ± 0.27
Andersson T, et al.[16]	9	6.7-12.5	1.4 ± 1.0	PO	NR	1.63 ± 0.39
Andersson T, et al.[16]	9	12.6-16.2	1.1 ± 0.3	PO	NR	1.47 ± 0.67
Faure C, et al. [17]	9	0.4-2.3	0.9 ± 0.3	IV	0.53 ± 0.29	NR
Present Study	23	2.0-16.0	0.4 ± 0.2	PO	1.76 ± 1.38	0.98 ± 0.22

Abbreviations include: n, number of subjects studied; IV, intravenous; PO, oral; NR, not reported; CL, apparent total plasma clearance (Cl/F for oral administration) and $t^{1}/_{2\lambda z}$, apparent terminal elimination half-life.
Data presented as mean ± SD generated from individual patient data presented in each of the cited investigations.
** denotes dose as mg/1.73m^2 of body surface area*

Similar to the findings from the previous intravenous studies, Andersson et al.[16] reported high variability in the AUC of omeprazole at steady state with dose-normalized values ranging from 0.1 to 27 μmol/l*hr in a population of 25 subjects (1.6 to 16.2 years of age) given doses ranging from 0.6 to 3.6 mg/kg. Considerable variability in $AUC_{0\rightarrow\infty}$ was also seen in our study cohort of 23 pediatric patients with values ranging from 236.9 to 1330.9 ng/ml*hr (Table 1). As expected, the primary source for this variability likely resided the fact that the actual weight-normalized dose of omeprazole administered to our subjects (i.e., 0.16 to 0.91 mg/kg) was quite different across the study cohort. This circumstance was unavoidable given that our study was limited to the use of the approved solid oral dosage form of the drug. Of further interest was that the median T_{max} values for omeprazole was similar between the previous[16] (i.e., 1.5 to 2 hours) and present (i.e., 2 hours; Table 1) study where the drug was given in both instances as either an intact capsule or the contents thereof sprinkled on applesauce (current study), yogurt or into fruit juice. While the previous study[16] suggested the presence of a possible age-dependence for elimination half-life, dose-normalized AUC and C_{max} for omeprazole, the authors did not identify a statistically significant association between age and any of the pharmacokinetic parameters. This is consistent with our findings and in particular, the lack of an observed association between the apparent terminal elimination rate constant (i.e., a pharmacokinetic parameter that is considered independent of drug absorption and thus, to reflect the processes responsible for drug clearance) and subject age (Figure 1).

Previous data pertaining to the pharmacokinetics of CYP2C19 substrates (e.g., phenytoin, diazepam) in pediatric patients summarized in a recent review suggests that the activity of this cytochrome P450 isoform may be significantly greater in infants and young children as compared to adolescents and adults.[25] Consequently, there was reason to suspect that the plasma clearance (and elimination half life) of omeprazole, a well characterized CYP2C19 substrate,[1,4] should be markedly different (i.e., increased clearance, shorter half life) in older infants and young children. However, a statistically significant relationship between subject age and omeprazole pharmacokinetics could not be demonstrated by the current or previous [15-17] investigations performed in pediatric patients (Table 2), despite an apparent direct trend demonstrated between age and AUC that suggested increased omeprazole clearance in younger patients.[16]

The previous pediatric pharmacokinetic investigations of omeprazole [15-17] did not evaluate the potential association of either the CYP2C19 phenotype or genotype with the pharmacokinetics of the drug.[1,5,6] As reported above, we did not find a statistically significant association between *CYP2C19* genotype (i.e., 1 vs. 2 functional alleles) and the apparent oral clearance, elimination rate constant or dose-normalized AUC (Figure 2) for omeprazole. Finally, our results for the 23 CYP2C19 extensive metabolizers (as defined by genotype) with respect to AUC comparison were in agreement with those reported from a recent adult study. This particular investigation demonstrated that while the omeprazole AUC following a single 20 mg oral dose in four subjects who were CYP2C19 poor metabolizers (5606.8 ± 1055.7 ng/ml*hr) was significantly different from that observed in both six homozygous extensive metabolizers (523.5 ± 120.4 ng/ml*hr) and five heterozygous extensive metabolizers (1095.6 ± 144.2 ng/ml*hr), there was not a statistically significant difference observed when the AUC values were compared between the homozygous and heterozygous extensive metabolizers.[6] While these data were admittedly not dose-normalized, they support our findings that suggest an apparent absence of a gene-dose effect for CYP2C19 extensive metabolizers relative to the pharmacokinetics of omeprazole.

Examination of the data from the current study failed to reveal two anticipated findings for omeprazole pharmacokinetics in pediatric subjects:

1. a demonstration of proportionality between a single oral dose of omeprazole and pharmacokinetic parameters reflective of systemic exposure (e.g., C_{max} and AUC) and

2. demonstration of an association between subject age and pharmacokinetic parameters that reflect drug elimination (e.g., Cl/F, elimination rate constant).

With regard to the assessment of dose-proportionality, a previous study of omeprazole pharmacokinetics following intravenous and oral administration of a single dose demonstrated apparent first-order kinetics for both omeprazole and the CYP2C19 catalyzed metabolite, 5'-hydroxyomeprazole.[23] This study did note a "slight dose-dependency" for omeprazole sulfone, a CYP3A4 catalyzed metabolite.[1] While admittedly indirect, our failure to demonstrate a significant association between the elimination rate constant for omeprazole and the apparent Cmax for the drug (Figure 3) supports that the pharmacokinetics of omeprazole in pediatric subjects, as in adults, appear to be first order (i.e., linear) following administration of a single oral dose. This is in contrast to the apparent nonlinearity in omeprazole pharmacokinetics with multiple dose administration that has been observed in several previous adult studies.[24]

Based on the results from the current study and the only previous investigation of omeprazole pharmacokinetics in pediatric patients following oral dosing,[16] it is likely that the high variability observed in the AUC obscured our ability to detect a relationship between dose and AUC and also, an age-association for either Cl/F and/or VDss/F; both of which are dependent upon AUC. In the absence of plasma concentration data for either 5'-hydroxyomeprazole or omeprazole sulfone, the likely causes for this variability cannot be directly addressed. Nonetheless, we speculate that potential reasons could well include compound-specific (i.e., as opposed to formulation-specific) interindividual and/or age-related differences in the extent of omeprazole oral bioavailability. As omeprazole biotransformation to the sulfone metabolite is catalyzed by CYP3A4[1] and also, the drug has been shown to be both a substrate and inhibitor of P-glycoprotein,[26] abundant expression of both proteins in the human small intestine, the great inter-subject variability in their activity (e.g., $\geq$ 20-fold) and the potential for age-associated up-regulation [27] would be expected to result in variable dose vs. plasma concentration (including AUC) relationships that could well be age-dependent. Unfortunately, previous pediatric pharmacokinetic studies of intravenous omeprazole in pediatric patients did not evaluate an oral dosing arm.[15,17] Hence, the absolute bioavailability of this drug in pediatric patients is not as yet known and may not be assumed to be identical to values (i.e., 35%

following single dose, approximately 60% following multiple doses) reported from previous investigations performed in adult subjects.[24]

It is also likely that the high variability in omeprazole systemic exposure prevented our ability to discern a difference between heterozygous and homozygous CYP2C19 extensive metabolizers for either dose-normalized AUC (Figure 2) or Cl/F. As suggested by previously summarized pharmacokinetic studies of pharmacologic substrates,[25,27] the activity of this cytochrome P450 isoform appears to be markedly increased in young infants and children as compared to adolescents and adults. If a true age-associated difference exists in the constitutive expression of activity for this enzyme in either the small intestine and/or liver, it is possible that its role in the overall metabolic profile for omeprazole in pediatric patients could markedly differ from adults, thus obscuring the relatively small (i.e., approximately 2-fold) difference in omeprazole exposure (assessed by comparison of mean AUC values) observed previously for subjects with 1 vs. 2 functional *CYP2C19* alleles.[6] As denoted above, the absence of metabolite data precludes our ability to "test" this assertion and hence, it remains both speculative and the subject for further study. Thus, for children and adolescents, neither age nor CYP2C19 phenotype in genotypically heterozygous extensive metabolizers appears to significantly influence the elimination half-life of omeprazole.

Finally, it should be noted that the range of systemic exposure to omeprazole reflected by the observed values for $AUC_{0\rightarrow\infty}$ (809.5 ± 893.8, 236.9 - 1330.9 ng/ml*hr) in our subjects who received an average 0.41 mg/kg (range – 0.16 to 0.91 mg/kg) single oral dose is in agreement with the range of AUC values (i.e., 223 to 1160 ng/ml*hr) reported from previous adult studies where single oral doses of omeprazole was administered and in several clinical treatment trials (i.e., with multiple dose administration), was shown to produce discernable, beneficial therapeutic effects.[24] This observation not only supports the apparent similarity for omeprazole pharmacokinetics between children and adults given a single oral dose of the drug but also suggests that an omeprazole dose of approximately 0.5 to 1.0 mg/kg should produce a degree of systemic exposure that in the majority of pediatric patients, could be expected to produce a therapeutic effect.[24]

In conclusion, the pharmacokinetics of omeprazole in children and adolescents between 2 and 16 years of age appear highly variable after oral administration of a single dose.

Pharmacokinetic studies following both oral and intravenous administration of the drug to date fail to provide convincing evidence of a predictable developmental dependence between either dose and systemic exposure (reflected by either C_{max} or AUC) or parameters that reflect drug elimination. Hence, the previous assertion that pediatric patients require weight-normalized doses of omeprazole which are higher than those considered to be "therapeutic" in adults does not appear to be supported by current pediatric pharmacokinetic data. To more completely assess the impact of *CYP2C19* pharmacogenetics and age on omeprazole disposition in infants and children, future investigations to assess both the absolute bioavailability of the drug and the complete metabolite profile (including stereo-specific analyses) will be required.

13. 6 Acknowledgement

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Chapter 14

Concordance between Tramadol and Dextromethorphan Parent/Metabolite Ratios: the Influence of CYP2D6 and non-CYP2D6 Pathways on Biotransformation

Based on the article:
Concordance between Tramadol and Dextromethorphan Parent/Metabolite Ratios: the Influence of CYP2D6 and non-CYP2D6 Pathways on Biotransformation
Abdel-Rahman SM, Leeder JS, Wilson JT, Gaedigk A, Gotschall RR, Medve R, Liao S, Spielberg SP and Kearns GL.
J Clin Pharmacol 42:24-29 (2002)

14.1 Abstract

Cytochrome P4502D6 (CYP2D6) activity has been shown to be a determinant of both the pharmacokinetics and pharmacodynamics of tramadol in adults. This study evaluated the association between CYP2D6 activity, as determined by dextromethorphan (DM) urinary metabolite ratio, and tramadol biotransformation in 13 children (age 7-16 years). CYP2D6 genotype was determined by XL-PCR and PCR/RFLP. Phenotype was assessed by HPLC quantitation of DM and its metabolites from a 12- to 24 hour urine collection following a single oral dose of DM. There was only a modest correlation between tramadol/M1 (metabolite 1) plasma concentration or AUC and the DM/dextrorphan (DX) urinary molar ratio in the study cohort; however, when subjects were segregated based on the number of functional CYP2D6 alleles, a much stronger relationship was observed for subjects with two functional alleles, with essentially no relationship evident in those individuals with one functional allele. Further evaluation of these data suggested that the CYP2D6-mediated metabolite (M1) is formed to a lesser extent, and the formation of the non-CYP2D6 product (M2) more pronounced in subjects with one versus two functional alleles. Thus, the number of functional CYP2D6 alleles and the availability of alternative cytochromes P450 capable of metabolizing tramadol may explain the poor association between DM, a well-characterized CYP2D6 probe, and tramadol in a population of CYP2D6 extensive metabolizers.

14.2 Introduction

The wide interindividual variability in drug concentrations observed in blood and urine, despite the administration of equivalent weight-based doses, can often be ascribed to variability in drug-metabolizing enzyme activity. Because of the impact that such wide fluctuations in drug concentration may have on the therapeutic response and toxicity of drugs with a narrow therapeutic index, the genotypic and phenotypic characterization of individuals and populations is increasingly undertaken in an attempt to predict enzyme activity and thus maximize drug safety and efficacy.[1]

For many drug-metabolizing enzymes, genotyping (e.g. identification of single nucleotide polymorphisms (SNPs), characterization of possible allelic variants) can enable successful

prediction of population phenotype (e.g. poor vs. extensive metabolizers). However, among individuals expressing functionally active protein, this technique may not readily distinguish quantitative differences in enzyme activity within a broad phenotypic classification where "normal" intersubject variability in the activity of an enzyme may be several fold.[2,3] Moreover, the predictive power of this tool can be compromised by several variables, including the number of alleles evaluated in a given genotyping procedure and the presence of variant alleles that remain uncharacterized, misclassified or unidentified.

Thus, to more fully characterize the functional consequence of any given SNP or allelic variant, pharmacologic phenotyping probes (i.e. drugs demonstrating a wide therapeutic index and negligible toxicity) are being increasingly used to quantitate differences in enzyme activity.[4] Ideally, the magnitude of turnover for a probe drug should correlate with the clearance and/or biotransformation of drug(s) of clinical interest that are substrates for the same enzyme. However, as with genotyping, the potential risk of misclassification still exists. The polyfunctionality of phenotyping probes used to measure activity of a P450 isoform,[5,6] the presence of an undetected drug-enzyme or drug-drug interaction,[7] or a phenotype that lies close to the population antimode can result in inconclusive or inaccurate assignment of enzyme activity. In fact, several studies to date demonstrate that phenotype assignment can correlate with concentration dependent drug toxicity and still fail to predict therapeutic response.[8]

This pilot study examined the association between dextromethorphan (DM), a well-established CYP2D6 phenotyping probe, and tramadol, a centrally acting analgesic that is biotransformed by this same polymorphically expressed P450 isoform (Figure 1). The O-demethylated metabolite (M1) of tramadol demonstrates an affinity for the μ-opioid receptor that is approximately 200 times greater than that of the parent compound. Thus, both the pharmacokinetic and pharmacodynamic activities of this analgesic are determined by CYP2D6 activity, as reflected by the genotype and resultant phenotypic expression of this enzyme.[9] Given this relationship, we hypothesized that the ratio between DM and its CYP2D6 product dextrorphan (DX) should reflect the turnover of tramadol to M1.

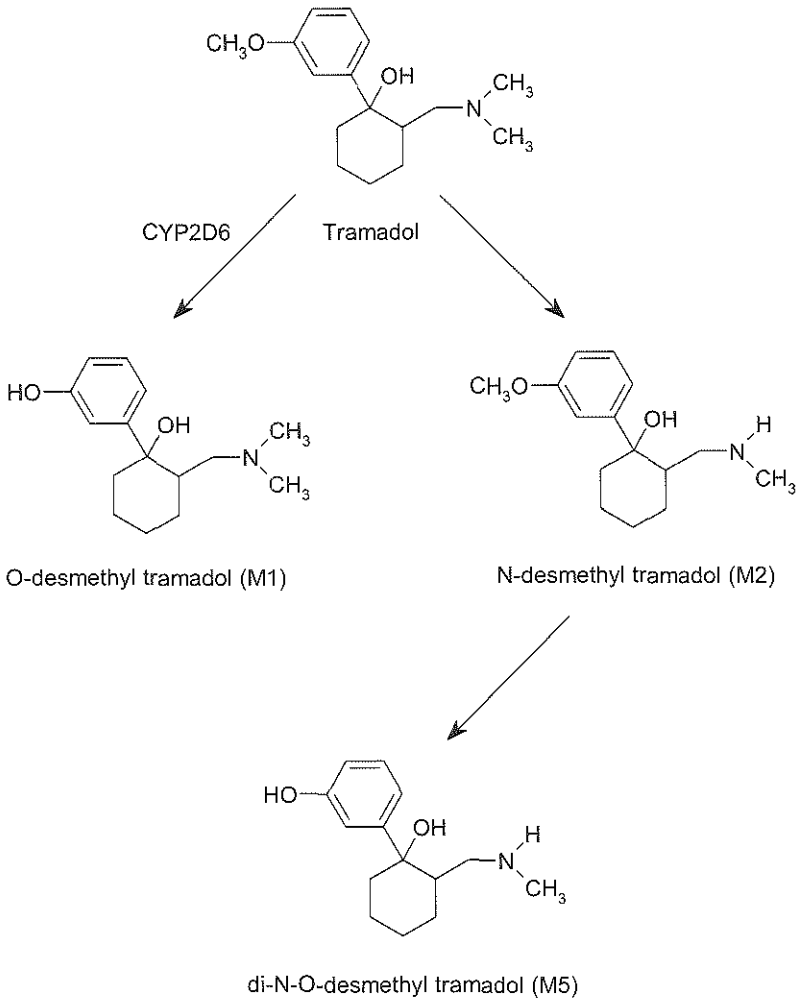

Figure 1 *Tramadol biodisposition pathway.*

14.3 Methods

Study Subjects

Subjects ($n = 26$) were recruited from concurrently running pharmacokinetic studies of tramadol conducted in children and adolescents between 7 and 16 years of age.[10] Children received a single body weight-adjusted dose of tramadol (Ultram® Tablets, Ortho-McNeil

Pharmaceuticals, Raritan, NJ) and six to eight blood samples were drawn over a 24-hour postdose period. Tramadol and metabolites (M1, M2 and M5) were determined in plasma by a validated LC/MS method.[11] Children receiving medication known to inhibit or induce any of the cytochromes P450 were excluded from participation. All subjects were enrolled via informed parental/guardian consent (and patient assent where applicable), and the study protocol was approved by the respective investigational review boards of the participating clinical institutions. In addition, the protocol was approved by the Network Steering Committee of the Pediatric Pharmacology Research Unit network.

Genotyping

CYP2D6 genotype was determined as described previously from leukocyte-derived genomic DNA isolated from whole blood.[12] An initial XL-PCR amplification generated a 5.1 kb-long product comprising the entire CYP2D6 coding region, which served as a template for subsequent PCR-RFLP assays to identify the *2, *3, *4, *6, *7, *8, *9, *10, *11, *12, *15, *17, *18 and *29 alleles according to standardized nomenclature (http://www.imm.ki.se/CYPalleles/). Additional XL-PCR reactions were performed to identify the *5 gene deletion as well as gene duplication alleles.

Phenotyping

Of the 26 children in whom CYP2D6 genotype was determined, 13 were subsequently phenotyped for CYP2D6 activity using DM urinary metabolite ratios. DM is preferentially (i.e., > 90%) metabolized by CYP2D6 to dextrorphan and has previously been validated as a marker for assessing the activity of this enzyme.[13] A single oral dose of DM (0.3 mg/kg as Tussin® Cough Syrup, Osco Drugs, Oak Brook, IL) was administered followed by a 12- to 24-hour urine collection. Total urine volume and pH of each quantitative urine collection were recorded. The urinary concentrations of DM and its metabolites – dextrorphan (DX), 3-methoxymorphinan (3MM), and 3-hydroxymorphinan (3HM) – were determined by HPLC with fluorescence detection as previously described.[14] The molar ratio of DM to DX was calculated and used as a surrogate measure of CYP2D6 activity, with a urinary DM to DX molar ratio of < 0.3 accepted as being indicative of an extensive metabolizer (EM) phenotype.[15]

Pharmacokinetic and Statistical Analyses

The log-linear trapezoidal rule was used to determine the AUC for tramadol and M1 from postdose plasma concentration versus time data. Least squares linear regression analyses were used to evaluate the association between CYP2D6 phenotype (i.e., DM/DX urinary molar ratio) and the postdose tramadol/M1 plasma concentration and AUC ratios at selected postdose time points. Further evaluation of the impact of CYP2D6 genotype on M1 formation was examined by construction of hysteresis curves of the mean plasma concentrations for tramadol and its M1 and M2 metabolites. Pharmacokinetic analyses were conducted using WinNonlin, version 1.5. Statistical analyses were conducted using SPSS for Windows version 9.0. The significance limit accepted for all statistical analyses was $\alpha = 0.05$.

14.4 Results

All subjects who were phenotyped with DM ($n = 13$) were genotypically consistent with a CYP2D6 extensive metabolizer phenotype. In 1 subject, a full genotype assignment was unavailable due to an inconclusive determination of the second allele. Thus, data from 12 children were available for this exploratory analysis. At all time points after maximal absorption (i.e., T_{max}) of single-dose tramadol administration, there was a modest correlation between tramadol/M1 plasma concentration or AUC and the DM/DX urinary molar ratio (correlation range: 0.43 - 0.56). However, when subjects were segregated based on the number of functional CYP2D6 alleles, a much stronger relationship was observed between tramadol and M1 concentration and AUC ratios and DM/DX urinary molar ratios in subjects with two functional alleles (correlation range: 0.77 - 0.90, $P < 0.01$). In contrast, essentially no relationship was evident between the aforementioned covariates in those individuals with one functional allele. No further significant relationships were found between the DM/DX ratio and select tramadol pharmacokinetic parameters most likely associated with the biotransformation of the parent drug (e.g., apparent oral clearance, elimination rate constant) regardless of segregation for the number of functional alleles.

To further evaluate whether the presence of one versus two functional CYP2D6 alleles had an apparent impact on the biotransformation of tramadol, the relationship of parent drug to metabolite concentration was examined as a function of CYP2D6 genotype.

Examination of the mean tramadol versus M1 plasma concentration profiles following a single dose demonstrated two distinct counterclockwise hysteresis curves for individuals with one versus two functional CYP2D6 alleles (Figure 2a). This figure suggests that M1 was formed to a lesser extent in individuals with one functional allele, and that for these individuals, CYP2D6 contributed to a lesser extent in overall tramadol biotransformation. Of interest was the finding that despite markedly lower M1 concentrations, average peak parent drug concentrations did not appear to differ significantly between subjects with one versus two functional alleles, indicating that an alternative metabolic pathway might account for an increased proportion of tramadol turnover. In fact, formation of the N-demethylated metabolite (M2) appeared to be more pronounced in subjects with one functional allele, accounting for a relative greater proportion of total metabolite formed (Figure 2b). Finally, examination of the AUC data revealed that less M5 was formed over the 24-hour post dose period in individuals with one functional allele (i.e., $AUC_{0\text{-}24}$: 92.8 ± 53.7 vs. 128.4 ± 61.4) despite the presence of more substrate available for this reaction. This is consistent with the aforementioned M1 data which suggested that tramadol O-demethylation appeared to be relatively rate limited in subjects with one functional CYP2D6 allele.

14. 5 Discussion

The results from this pilot pharmacokinetic-pharmacogenetic study of a therapeutic CYP2D6 substrate provide new and potentially valuable insights regarding the interpretation of pharmacogenetic data obtained as part of a clinical trial. The initial presence of only a weak association between tramadol and DM biotransformation in our subject cohort was certainly curious and was thought to be consequent to the inter-individual variability in urinary DM/DX molar ratios which normally span more than three orders of magnitude in individuals classified as extensive metabolizers or indeed, who have the same CYP2D6 genotype (e.g., *1*1).[12] More intriguing, however, was the appreciation of a strong relationship between the DM/DX and tramadol/M1 ratios in individuals with two functional CYP2D6 alleles and the virtual disappearance of this relationship in those individuals with one functional allele.

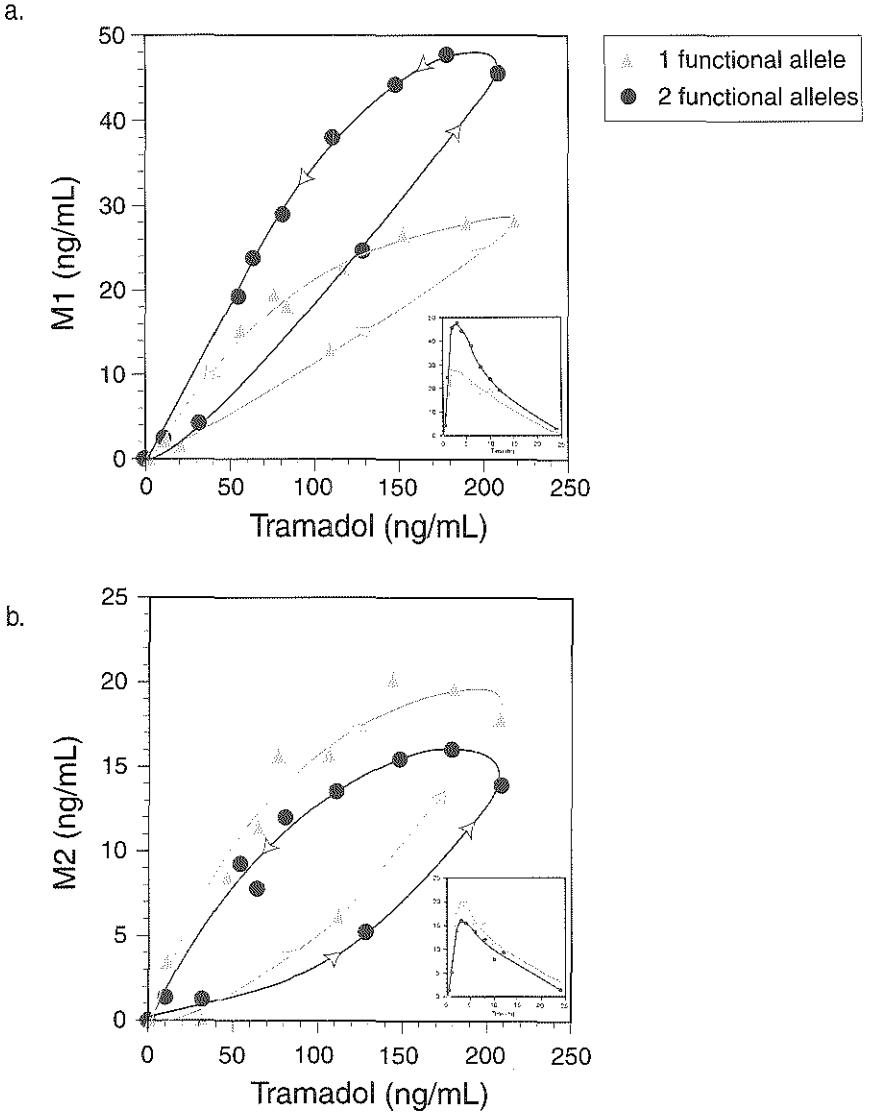

Figure 2 *(a) Hysteresis plot looking at formation of M1 from tramadol in subjects with one versus two functional alleles. Inset shows mean postdose plasma M1 concentration versus time data for both groups. (b) Hysteresis plot looking at formation of M2 from tramadol in subjects with one versus two functional alleles. Inset shows mean postdose plasma M2 concentration vs. time data for both groups.*

Although we only examined data from a small number of children, our results suggest that CYP2D6 phenotype, as characterized by the molar urinary ratio of DM/DX, is less predictive of the extent of tramadol O-demethylation in individuals with one versus two functional CYP2D6 alleles. The poor correlation in the group of individuals with one functional allele may be due to sole reliance on the protein product of a single functional allelic variant which itself may have reduced capacity to turnover substrates due either to mutations in the coding region affecting catalytic activity or mutations in regulatory regions affecting the level of gene expression.[16,17]

It is also possible that alternative metabolic pathways may play a more pronounced role in the biotransformation of tramadol for individuals with one functional CYP2D6 allele. Although there was no apparent association between the number of functional alleles and the disappearance of tramadol from plasma given that the mean plasma concentration versus time curves essentially overlay one another in these two populations, the same cannot be said for the primary metabolites. The mean plasma concentration curves that illustrate the appearance or formation of metabolite (e.g., M1 and M2, Figure 2) do appear to discriminate between individuals with one versus two functional CYP2D6 alleles. In situations where the disposition of a compound is solely dependent upon a single biotransformation pathway, one would expect pharmacogenetic differences in metabolism to be reflected by pharmacokinetic data for the parent drug. However, in situations where two or more enzymes are capable of catalyzing the biotransformation of a specific substrate, the relative contribution of each pathway to the overall disposition of the compound is a function of the individual patient's unique complement of drug biotransformation pathways, including the constitutive expression of the drug-metabolizing enzymes.

The data from our pilot study also provide corroborating evidence for a possible gene dose effect for CYP2D6 that has to date been alluded to only in the characterization of the disposition of probe compounds.[18,19] Given the size limitations of our study cohort, the potential clinical significance of this finding as relates to tramadol disposition remains to be determined. However, for other CYP2D6 substrates that have a low therapeutic index (e.g., antidepressants, antiarrhythmics, neuroleptics, beta-adrenoceptor antagonists), the number of functional allelic variants of CYP2D6 in extensive metabolizers, along with the presence or absence of secondary metabolic pathways, may translate to clinical

importance, especially when biotransformation results in bioactivation or the formation of metabolites with therapeutic and/or toxic potential.

In conclusion, the biotransformation of tramadol and perhaps other CYP2D6 substrates may vary within the phenotypic population of extensive metabolizers for this cytochrome P450 isoform. Accordingly, when pharmacogenetic data are examined to assess population-specific differences in drug disposition, it is imperative that a complete pharmacogenetic assessment be coupled with a detailed evaluation of drug metabolites, especially those that are produced by the drug-metabolizing enzyme under study.

14. 6 Acknowledgement

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Chapter 15

Summary, Discussion and Future Directions

15.1 Preface

Development *per se* represents a continuum of biologic events that enable adaptation, somatic growth and eventually, reproduction. From a societal, psychosocial, behavioral and medical perspective, it is generally appreciated that infants and children are far different from adults and elderly. As well, pediatric patients suffer from conditions and diseases that in many instances are unique and have no true counterpart in adults. These particular differences are generally known and to a great degree, determine how infants, children and adolescents are provided for both within the context of medical care and in society. What remains often under-appreciated is the fact that development exerts profound influences on pediatric pharmacotherapy. Specifically, normal human development is a dynamic process, especially during the first five years of life, with changes in organ structure and function that can dramatically impact upon drug disposition and action. Unlike the different sizes of infants and children that are readily apparent from visual inspection, the impact of ontogeny on pharmacokinetics and pharmacodynamics of therapeutic drugs and other xenobiotics is often not apparent.

The basic and translational science investigations that constitute this thesis were performed specifically to examine the impact of ontogeny on the activity of important drug metabolizing enzymes. This was accomplished using "traditional" approaches (e.g., examining the pharmacokinetics of a specific drug known to be a substrate for a specific enzyme as a function of age), by developing new techniques with enhanced specificity suitable for use across the pediatric age spectrum (e.g., characterization of cisapride as a selective CYP3A4 probe) and by demonstrating how integration of the "new biology" (e.g., incorporation of relevant pharmacogenomics) into pediatric clinical pharmacology investigations can be used as a "tool" to examine how much of the variability observed in pediatric pharmacokinetic data is associated with development vs. normal, genetically determined interindividual variability in the activity of a drug metabolizing enzyme.

15. 2 Summary

Introduction

Chapter 1 provides a historical introduction to Pediatric Clinical Pharmacology by

reviewing its origins and the recent developments that have produced a true renaissance in the discipline. This chapter also outlines the goals of the research conducted as part of this thesis.

Chapter 2 represents a comprehensive, state-of-the-art review of Pediatric Clinical Pharmacology. It systematically examines the impact of ontogeny on drug disposition and action and provides specific examples of how developmental differences in drug absorption, distribution, metabolism, excretion and pharmacodynamics influence pediatric pharmacotherapy. Finally, this chapter provides a prospective on the importance of including infants, children and adolescents as subjects in clinical trials so that developmental differences in pharmacokinetics and pharmacodynamics can be effectively translated via the drug development process into information that can be effectively utilized by the physician to improve the safety and efficacy of pediatric drug therapy.

The Impact of Development on Pharmacokinetics

Chapter 3 describes the results from a traditional pharmacokinetic study designed to examine the impact of development on the disposition of metoclopramide, a prokinetic drug used extensively in neonates and young infants, that is metabolized by the polymorphically expressed enzyme, CYP2D6. In this study of 10 premature infants (1.1 to 3.2 kg, 31 to 40 weeks postconceptional age), considerable variability in the apparent plasma clearance of metoclopramide was seen, with 30% of the infants having a markedly prolonged elimination half life (i.e., > 10 hours). This proportion was considerably greater than the incidence of the poor-metabolizer phenotype for CYP2D6 (e.g., 2-7%) and thus, was reflective of a developmental reduction in activity for CYP2D6 and potentially, other phase II enzymes important in the clearance of metoclopramide (e.g., sulfotransferases) in selected infants.

Chapters 4 and 5 illustrate that development can exert profound influences on the clearance of a pharmacologic substrate that is not metabolized by cytochromes P450 or other phase II drug metabolizing enzymes (e.g., glucuronosyltransferases, sulfotransferases, methyltransferases). An initial pharmacokinetic study of linezolid, a new oxazolidinone antimicrobial agent, was conducted in a cohort of 58 children, ranging in age from 3 months to 16 years (**Chapter 4**). Plasma clearance data obtained following intravenous administration of either a 1.5 or 10 mg/kg dose of the drug was significantly

associated with age, with those infants who were less than 40 months of age demonstrating the highest clearance values. Examination of renal clearance data for the study cohort revealed that the age-dependence in linezolid pharmacokinetics was a function of non-renal clearance. The potential clinical significance of these developmental differences in pharmacokinetics was translated to age-specific dosing recommendations for linezolid by projection of steady state plasma concentrations (as a function of age-specific pharmacokinetic data) and comparison to the average *in vitro* susceptibility profiles for selected pathogens. Given the potential utility of linezolid in treating neonatal infections caused by methicillin-resistant pathogens (e.g., MRSE, MRSA), the aforementioned pharmacokinetic investigation was extended to a population of 42 neonates and young infants that was stratified on the basis of both postnatal (i.e., less than and greater than or equal to 8 days) and gestational (i.e., less than and greater than or equal to 34 weeks) age (**Chapter 5**). In this study, linezolid plasma clearance was also found to be associated with age, with dramatic differences (i.e., and approximate 3-fold increase) seen during the first week of life irrespective of gestational age. Thus, these two studies collectively demonstrated a clear biphasic developmental pattern for linezolid plasma clearance. Given that linezolid is extensively biotransformed by ubiquitously expressed non-cytochrome P450 enzymes (e.g., esterases), the pharmacokinetic data for this drug across the pediatric age spectrum provides evidence for rather dramatic developmental expression of enzymes that are not characteristically viewed as important for the biotransformation of most therapeutic drugs.

The Impact of Ontogeny on the Activity of CYP3A4

The cytochrome P450 3A subfamily is the most abundantly expressed of all cytochromes P450 in the human body and collectively, is responsible for regulating the biotransformation of over 100 known therapeutic drugs and xenobiotics. **Chapter 6** reviews the relevant biology of human CYP3A and multiple examples from the clinical pharmacology literature of how the pharmacokinetics of substrates for these enzymes reveal a "pattern" of developmental expression for three of the most well studied isoforms; CYP3A4, CYP3A5 and CYP3A7.

Chapters 7 and 8 are complimentary in that they describe the results from two pharmacokinetic studies of a pharmacologic substrate for CYP3A4/5; namely, midazolam. This benzodiazepine is extensively used in pediatric medicine for its sedative

and anxiolytic properties and its short duration of action produced largely by rapid clearance from plasma. Clinical pharmacogenetic investigations conducted in the U.S., Canada and Europe over the last decade have generally accepted midazolam (over dapsone and the erythromycin breath test) as the pharmacologic probe "of choice" to assess the activity of CYP3A4/5 *in vivo*. **Chapter 7** reports the results from a pharmacokinetic study of midazolam conducted in a total of 133 pediatric patients who ranged in age from 6 months to 16 years performed as part of a development program to secure approved pediatric labeling of the drug in the U.S. Significant developmental differences were noted for both the absorption of midazolam and its plasma clearance, the latter being substantially greater in the younger children (i.e., those < 12 years of age). Examination of the pharmacokinetic data for the primary metabolite of midazolam (i.e., α-hydroxymidazolam) demonstrated the greatest formation in the youngest subjects and *vide inferre*, higher CYP3A4/5 activity.

Chapter 8 describes the results from a pharmacokinetic study of midazolam and α-hydroxymidazolam following a 0.1 mg/kg intravenous dose to 24 preterm infants (26 to 34 weeks gestational age, 3 to 11 days postnatal age). While no significant association was observed between age (gestational, postnatal or postconceptional) and midazolam plasma clearances consequent to significant interindividual variability (i.e., approximately 10-fold), the average value for plasma clearance in the preterm infants was substantially less than that observed from the prior study conducted in older infants, children and adolescents (**Chapter 7**); thus demonstrating a marked developmental reduction in CYP3A4/5 activity. This was corroborated by examination of the data reflecting the apparent formation of the major metabolite (i.e., α-hydroxymidazolam). An incidental finding from this investigation was the apparent effect of concomitant indomethacin therapy (for treatment of patent ductus arteriosus) on midazolam disposition; thus suggesting an interaction between both development and the treatment of disease on the expression of CYP3A4/5 activity during the first two weeks of life.

Development of a Selective Pharmacologic Probe for CYP3A4

Previous investigations attempting to use pharmacologic probes to assess the activity of CYP3A4 were confounded by limitations of compounds being used. In some instances, the probe compounds were polyfunctional substrates for cytochromes P450 (e.g., dapsone, dextromethorphan) and thus, were not selective for CYP3A4. In the case of

erythromycin, it was found to be actively transported by P-glycoprotein (P-gp) and thus, its disposition profile did not accurately reflect the activity of a single enzyme. Midazolam, a compound studied as part of this thesis (**Chapters 7 and 8**), does provide an accurate estimate of total CYP3A activity as it is a substrate for both CYP3A4 and CYP3A5, the later isoenzyme being expressed to a significant degree in human liver with an apparent association with race. Thus, a selective pharmacologic probe to assess CYP3A4 activity was not available which virtually prevented the evaluation of the impact of ontogeny on this particular isoform *in vivo*.

Chapter 9 describes the results from the first in a series of investigations to validate the use of cisapride as a selective pharmacologic probe compound for assessing the activity of CYP3A4. Reaction phenotyping was performed using phenotyped human liver microsomes and microsomes prepared from baculovirus-infected insect cells that expressed ten different human cytochromes P450 including CYP3A4, 3A5 and the fetal form, CYP3A7. The biotransformation of cisapride to norcisapride and two major metabolites, 4-Fluoro-2-hydroxycisapride (4F) and 3-Fluoro-4-hydroxycisapride (3F) was examined using a novel HPLC/MS method. As well, a series of studies were performed using well-characterized pharmacologic inhibitors of selected cytochromes P450. Collectively, these studies demonstrated that the formation of norcisapride and the major cisapride metabolites were consistent with Michaelis-Menten kinetics for a single enzyme. Studies with cDNA-expressed enzymes revealed that CYP3A4 catalyzed the biotransformation of cisapride (at concentrations similar to those observed in human plasma during therapy with the drug) to norcisapride and 4F at rates > 100 times those of non-CYP3A enzymes and > 100- and 50-fold higher than CYP3A5 and CYP3A7, respectively. Thus, it was apparent that at "therapeutic" cisapride concentrations, CYP3A4 was primarily and predominantly responsible for the metabolism of this drug with contributions from other cytochromes P450 being quantitatively not important. Accordingly, the *in vitro* investigations demonstrated that cisapride was a candidate pharmacologic probe to assess CYP3A4 activity.

The next steps in evaluating cisapride as a pharmacologic probe were to determine whether the drug was transported by P-gp and to examine for the presence of concordance when selected pharmacokinetic parameters (e.g., plasma clearance, AUC) were compared *in vivo* to a previously characterized CYP3A4 probe compound. **Chapter 10** presents data

from an *in vitro* and *in vivo* study of cisapride. Bi-directional transport assays were conducted in LLC-PK1 cells and the derivative cell line L-MDR1 to determine whether cisapride was a substrate for P-gp. While not a substrate for this enzyme, cisapride did inhibit P-gp with an apparent Ki of 16.1 μM. At the conclusion of this study, it was apparent from *in vitro* data that cisapride possessed the requisite selectivity to be pursued as a selective pharmacologic probe for CYP3A4. Accordingly, an *in vivo* study was performed in 17 healthy adults who, on separate occasions, received a single intravenous (0.025 mg/kg) and oral (0.15 mg/kg) dose of midazolam, and an oral dose (0.07 mg/kg) of cisapride, followed in each instance by the performance of a pharmacokinetic study. From this investigation, statistically significant linear correlations were found between the cisapride apparent oral clearance and the clearance of both intravenous and oral midazolam, thus demonstrating concordance between these two putative CYP3A4 pharmacologic probes. As well, the data demonstrated that the apparent plasma clearance and AUC for cisapride could be accurately and reliably predicted from a single 8 or 12-hour post-dose plasma concentration measurement. The results from these preliminary investigations of cisapride (**Chapters 9 and 10**) coupled with the world-wide clinical experience with this drug in neonates and infants suggested that cisapride could be used as a selective probe to assess total CYP3A4 activity and to study the true impact of ontogeny on this cytochrome P450 isoform.

The results from a pharmacokinetic investigation of cisapride designed to assess the ontogeny of CYP3A4 *in vivo* are presented in **Chapter 11**. In this investigation, 35 infants, 30-51 weeks postconceptional age (PCA), received a single oral dose (0.2 mg/kg) of cisapride at 4 to 87 days of postnatal age. Plasma concentrations of both cisapride and norcisapride were quantitated and used to derive pharmacokinetic parameter estimates. As well, cisapride renal clearance was estimated. In all infants, non-renal clearance of cisapride accounted for virtually all of the apparent plasma clearance of the drug. Sub-stratification of the population demonstrated an apparent plasma clearance (0.45 L/hr/kg) in the youngest, most immature infants (i.e., 28 – 36 weeks PCA) that was approximately half of that seen in infants > 36 weeks PCA. Also, a positive, linear correlation was found between the apparent terminal elimination rate constant for cisapride and PCA. Examination of ratios for the AUC of norcisapride : cisapride revealed an extent of variability similar to that previously reported for the normal interindividual variability for CYP3A4 in adults (i.e., approximately 25-fold). Examination of the pharmacokinetic data

for cisapride as a function of age demonstrated that a dramatic increase in CYP3A4 activity in the first three months of life is apparent, thus providing *in vivo* evidence for the impact of ontogeny on this particular cytochrome P450 isoform.

Integrating Pharmacogenetics into Studies to Assess the Impact of Age on Drug Disposition

Chapter 12 provides a review and commentary regarding the need and challenges of delivering pharmacogenomics into clinical pediatrics. The application of pharmacogenetics and pharmacogenomics in a developmental context is discussed as are the challenges presented by pediatric disease states (including drug-induced conditions) and the development of relevant model systems. Additionally, special ethical and scientific considerations are presented that could, if executed poorly, limit the application of pharmacogenomics or alternatively, if done properly, could make the investigation of drugs in pediatrics more robust. Proof-of-concept for this latter assertion is provided by **Chapters 13 and 14** where relevant pharmacogenetics was integrated into two clinical trials conducted in pediatric patients as part of a formal drug development program.

Omeprazole, a proton pump inhibitor and substrate for the polymorphically expressed enzyme CYP2C19, is routinely used in infants, children and adolescents in the U.S., Canada and Europe to treat gastroesophageal reflux disease and a variety of other conditions where control of intragastric pH is necessary. **Chapter 13** presents results from a pharmacokinetic study of this compound conducted in 37 children and adolescents from 2 to 16 years of age who received a single oral dose (10 or 20 mg) of the drug. Because the pharmacokinetics and pharmacodynamics of omeprazole in adults have been shown to be dramatically influenced by CYP2C19 phenotype, we included CYP2C19 genotyping (an indicator of phenotype) as a component of the study in an attempt to: 1). describe potential "outliers" for the pharmacokinetic data and 2). examine how much of the potential variability in omeprazole disposition across the study cohort was associated with age vs. pharmacogenetic (i.e., CYP2C19 genotype) determinants. In contrast to previous assertions by other investigators, we found no age dependence in the disposition of omeprazole in pediatric patients between 2 and 16 years of age. The degree of systemic exposure associated with the 10 to 20 mg doses of omeprazole in children was similar to that observed in adults taking therapeutic doses of the drug. Only one CYP2C19 poor-metabolizer was identified in the study cohort who, as expected, had AUC and peak

plasma concentration values that were significantly higher than those found in the subjects with a genotype consistent with a CYPC19 extensive metabolizer phenotype. Examination of the pharmacokinetic data for omeprazole revealed substantial variability for both the AUC and apparent oral plasma clearance, thus prompting us to evaluate whether a potential gene-dose effect was apparent. However, comparison of mean AUC values normalized for dose (i.e., per 1 mg/kg) between subjects with 1 vs. 2 functional CYP2C19 alleles revealed no statistically significant difference.

Chapter 14 describes the results from a pediatric clinical pharmacokinetic study of tramadol, a non-opiate analgesic agent that is a substrate for the polymorphically expressed enzyme, CYP2D6. This investigation entailed an evaluation of the plasma disposition profile for tramadol and its metabolites (M1 and M2) in 26 children (7 to 16 years) who received a single oral dose of the drug. A subset of the study cohort (n = 13) had CYP2D6 genotype determined and also, assessment of phenotype by quantitation of dextromethorphan (DM) and its primary metabolites (dextrorphan, 3-methoxymorphinan and 3-hydroxymorphinan) from urine following a single oral dose of DM. In this study, no apparent age-dependence in tramadol pharmacokinetic parameters was noted. There was, however, a modest correlation between the tramadol/M1 plasma concentration or AUC ratio and the DM/dextrorphan urinary molar ratio. However, when the subjects were segregated based upon the number of functional CYP2D6 alleles, a much stronger relationship was found in the subjects with two (as opposed to one) functional alleles. Further evaluation of the data suggested that the CYP2D6 mediated metabolite (M1) is formed to a lesser extent and the formation of the non-CYP2D6 product (i.e., the M2 metabolite) was more pronounced in subjects with one vs. two functional alleles. Thus, a potential gene-dose effect for CYP2D6-mediated drug biotransformation was evident.

15.3 Future Directions

Despite tremendous advances in the discipline of Pediatric Clinical Pharmacology made over the past decade, significant information gaps remain with respect to our ability to completely and in many cases, accurately characterize the impact of ontogeny on the activity of important drug metabolizing enzymes. As in the past, pharmacokinetic studies of therapeutic drugs are limited in their utility for this purpose as the vast majority are

metabolized by a host of different enzymes (i.e., are polyfunctional substrates). Accordingly, the "picture" of ontogeny that these investigations provide is limited in scope and therefore, is incomplete.

The post-genome era presents unprecedented opportunities to make significant contributions to clinical pediatrics through the purposeful and prudent incorporation of pharmacogenetic and/or pharmacogenomic "tools" into clinical trials designed to examine drug disposition, action and efficacy. As we have shown, this can markedly enrich a pharmacokinetic study by providing evidence sufficient to explain the appearance of an extreme discordant phenotype and in investigations designed to assess the impact of ontogeny on drug disposition, provide a means to dissect the influence of development *per se* from the normal, expected interindividual variability (in drug metabolizing enzyme activity) that to a great degree, is under genetic control. Thus, the inclusion of relevant pharmacogenetics (or pharmacogenomics) in a clinical pharmacokinetic study can effectively bring the data "to life" and thereby, enhance both their interpretation and ultimate clinical utility.

Two new frontiers for the interface between ontogeny and pharmacogenetics are apparent. The first resides with identifying the "triggers" that produce age-associated changes in the activity of drug metabolizing enzymes. This will require a detailed study of events that control gene expression such as xenobiotic interactions with polymorphically expressed nuclear receptors, repression and/or de-repression of post-receptor elements in the gene promoter region and their collective interaction with polymorphisms in the promoter and/or regulatory regions of candidate genes. The second resides with exploring the pharmacogenetic determinants of drug action. This will require establishing roles for specific genes, single nucleotide polymorphisms or haplotypes in disease etiology or treatment outcome. Finally, for the revelations associated with this era of the human genome to be translated into discoveries with the potential to favorably impact the care and treatment of children, it will be incumbent upon society (i.e., both the scientific and lay public) to embrace the multiple opportunities afforded by this new knowledge and make every effort to integrate it into the development of drugs critical to promote the health of pediatric patients.

15.4 Samenvatting

Ontwikkeling is een continuüm van biologische gebeurtenissen dat leidt tot adaptatie, lichamelijke groei en uiteindelijk tot voortplanting. Vanuit maatschappelijk, psychosociaal, gedragsmatig en medisch perspectief wordt algemeen aanvaard dat zuigelingen, peuters, kleuters en grotere kinderen sterk verschillen van volwassenen en bejaarden. Kinderen lijden vaak aan ziekten die in veel gevallen uniek zijn voor de kinderleeftijd en daardoor bij volwassenen niet worden gezien. Deze verschillen bepalen tot in hoge mate hoe zuigelingen, peuters, kleuters, grotere kinderen en adolescenten door de medische wereld en de maatschappij worden "behandeld". Wat vaak onderbelicht blijft is het feit dat ontwikkeling ook een zeer belangrijke invloed uitoefent op farmacotherapie bij kinderen. Meer specifiek, normale menselijke ontwikkeling is een dynamisch proces met name gedurende de eerste vijf levensjaar, met daarmee gepaard gaande veranderingen in orgaanstructuur en –functie met dramatische gevolgen voor de verdeling en werking van geneesmiddelen. Anders dan fysieke verschillen (lengte, gewicht etc.) tussen zuigelingen, peuters, kleuters en oudere kinderen die op het eerste oog duidelijk zijn is het effect van (ontwikkeling) ontogenie op de farmacokinetiek en –dynamiek van geneesmiddelen en xenobiotica vaak helemaal niet zo duidelijk.
De basale en translationele research projecten waaruit dit proefschrift bestaat werden uitgevoerd om specifiek het effect van de ontogenie op de activiteit van belangrijke enzymen voor het metabolisme van geneesmiddelen te onderzoeken. Dit werd bereikt door gebruik te maken van "klassieke" benaderingen zoals het bestuderen van de farmacokinetiek van een specifiek geneesmiddel dat bekend stond als een substraat voor een specifiek enzym als een functie van de leeftijd. Het ontwikkelen van nieuwe technieken met toegenomen specificiteit die te gebruiken zijn bij kinderen van alle leeftijden zoals het karakteriseren van cisapride als een selectief CYP3A4 modelmedicijn en door het aantonen van het nut van integratie van de zogenaamde "nieuwe biologie", namelijk het incorporeren van de farmacogene nieuwe informatiebron dat in klinische farmacologie bij kinderen gebruikt kan worden als een stuk gereedschap om te onderzoeken hoeveel van de gevonden variabiliteit in farmacokinetische gegevens geassocieerd is met ontwikkeling dan wel past bij de normale, genetisch bepaalde interindividuele variabiliteit in de activiteit van het desbetreffende enzym.

Introductie

Hoofdstuk 1 geeft een historisch overzicht van de klinische kinderfarmacologie vanaf het eerste begin tot aan de meeste recente ontwikkelingen die hebben geleid tot een ware wedergeboorte van het vakgebied. Dit hoofdstuk geeft tevens een opsomming van de verschillende studiedoelen van dit proefschrift.

Hoofdstuk 2 is een bondig overzicht van de klinische farmacologie bij kinderen. Op een systematische wijze wordt enerzijds de invloed van ontogenie op geneesmiddeldispositie en -actie beschreven en anderzijds worden specifieke voorbeelden gegeven die laten zien dat deze ontwikkelingsafhankelijke veranderingen in absorptie, -distributie, -metabolisme, -excretie en farmacodynamiek van geneesmiddelen een grote invloed hebben op het voorschrijven van geneesmiddelen aan kinderen. Tot slot geeft dit hoofdstuk het belang aan zuigelingen, peuters, kleuters, oudere kinderen en adolescenten te includeren in klinische studies opdat ontwikkelingsafhankelijke verschillen in farmacokinetiek en –dynamiek op een effectieve wijze vertaald kunnen worden tijdens het proces van geneesmiddelontwikkeling. Deze informatie kan op een effectieve wijze gebruikt worden door de voorschrijvend arts om de veiligheid en effectiviteit van de farmacotherapie bij kinderen te verbeteren.

De rol van ontwikkeling op farmacokinetiek

Hoofdstuk 3 beschrijft de resultaten van een klassieke farmacokinetische studie die ontworpen werd om het effect van ontwikkeling op de dispositie van metoclopramide, een prokineticum dat zeer frequent wordt gebruikt bij pasgeborenen en zuigelingen en dat gemetaboliseerd wordt door CYP2D6, een enzym met polymorfe expressie, te onderzoeken. In deze studie bij 10 preterme pasgeborenen (1.1 - 3.2 kg, 31 - 40 weken postconceptionele leeftijd) werd een aanzienlijke variatie in de plasmaklaring van metoclopramide gezien, waarbij bij 30 % van de bestudeerde kinderen een duidelijk verlengde halfwaardetijd (> 10 uren) werd gezien. Dit percentage was aanzienlijk groter dan het voorkomen van het zogenaamde "poor-metabolizer" fenotype voor het enzym CYP2D6 (2 – 7 %). Dit toonde aan dat er een ontwikkelingsafhankelijke reductie in CYP2D6 activiteit is en mogelijk ook van andere, fase II enzymen, die van belang kan zijn voor de klaring van metoclopramide (b.v., sulfotransferases) bij sommige kinderen.

Hoofdstuk 4 en 5 illustreren dat ontwikkeling een grote invloed kan uitoefenen op de klaring van een farmacologisch substraat dat niet door cytochroom P450 of andere, fase II geneesmiddel metaboliserende enzymen (b.v., glucuronosyltransferases, sulfotransferases, methyltransferases) wordt omgezet. Een eerste farmacokinetische studie met linezolid, een nieuw oxazolidinon antimicrobieel geneesmiddel, werd uitgevoerd bij een cohort van 58 kinderen variërend in leeftijd van 3 maanden tot 16 jaar (***Hoofdstuk 4***). Data betreffende de plasmaklaring na intraveneuze toediening in een dosis van 1.5 mg/kg of 10 mg/kg respectievelijk waren significant geassocieerd met leeftijd waarbij kinderen met een leeftijd jonger dan 40 maanden de hoogste klaring lieten zien. Onderzoek van de data betreffende de renale klaring van linezolid bij deze kinderen toonde aan dat de leeftijdsafhankelijkheid van de farmacokinetiek van linezolid als een functie van de niet-renale klaring moet worden beschouwd. Het belang voor de kliniek van deze ontwikkelingsafhankelijke verschillen in farmacokinetiek werd vertaald naar leeftijdspecifieke doseringsadviezen voor linezolid waarbij gebruik werd gemaakt van berekende steady state plasma concentraties (als een functie van de leeftijdspecifieke farmacokinetiek) en *in vitro* gevoeligheidsprofielen van de geselecteerde ziekteverwekkers. Rekening houdend met de potentiële bruikbaarheid van linezolid voor de behandeling van neonatale infecties veroorzaakt door methicilline resistente pathogenen (MRSE, MRSA), werd de bovengenoemde farmacokinetische studie uitgebreid naar een groep van 42 pasgeborenen en zuigelingen die gestratificeerd werden op basis van zowel leeftijd (jonger, gelijk aan of ouder dan 8 dagen) en zwangerschapsduur (jonger, gelijk aan of ouder dan 34 weken) (***Hoofdstuk 5***). In laatstgenoemde studie was de plasmaklaring van linezolid wederom geassocieerd met leeftijd waarbij dramatische verschillen (een bijna drievoudige toename in de klaring) werden gezien in de eerste levensweek onafhankelijk van zwangerschapsduur. Deze twee studies lieten derhalve een duidelijk bifasisch ontwikkelingspatroon zien voor de plasmaklaring van linezolid. Gegeven het feit dat linezolid intensief wordt gemetaboliseerd door de ruim aanwezige niet cytochroom gebonden P450 enzymen (b.v. esterases), blijkt uit de farmacokinetische gegevens van dit geneesmiddel op de verschillende kinderleeftijden dat zeer grote veranderingen plaatsvinden in de expressie van enzymen die van oudsher niet van belang werden geacht voor het biotransformeren van geneesmiddelen.

De rol van ontogenie op de activiteit van CYP3A4
De cytochroom P450 3A subfamilie komt van alle P450 cytochromen het meest tot expressie in het menselijk lichaam en is gezamenlijk verantwoordelijk voor het reguleren van de biotransformatie van meer dan 100 bekende geneesmiddelen en xenobiotica.

Hoofdstuk 6 geeft een overzicht van de relevante biologie van het menselijk CYP3A en geeft vele voorbeelden vanuit de klinische farmacologie literatuur over de wijze waarop farmacokinetiek van substraten voor deze enzymen een, op ontwikkeling gebaseerd, patroon van expressie onthullen voor drie van de best bestudeerde isoformen: CYP3A4, CYP3A5 en CYP3A7.

Hoofdstuk 7 en 8 vullen elkaar aan omdat de resultaten van twee farmacokinetische studies van een farmacologisch substraat van CYP3A4/5 beschreven worden, namelijk midazolam. Dit benzodiazepine wordt uitgebreid gebruikt in de kindergeneeskunde van wegen de sedatieve en angstverlagende werking en de korte werkingsduur die veroorzaakt wordt door de snelle plasmaklaring. Klinische studies die de afgelopen tien jaar uitgevoerd zijn in de Verenigde Staten, Canada en Europa hebben ertoe geleid dat midazolam (en niet dapsone of de erythomycine ademtest) wordt gezien als het modelmedicijn van eerste keuze om CYP3A4/5 activiteit *in vivo* te meten.

Hoofdstuk 7 geeft de resultaten weer van een farmacokinetische studie met midazolam uitgevoerd bij 133 kinderen die in leeftijd varieerden van 6 maanden tot 16 jaar als onderdeel van een programma dat als doel had de juiste plaatsbepaling voor midazolam als een geneesmiddel voor kinderen in de Verenigde Staten te verkrijgen. Significante ontwikkelingsafhankelijke verschillen werden gezien in zowel absorptie van midazolam als plasmaklaring. Laatstgenoemde was duidelijk sneller bij jongere kinderen (degene met een leeftijd van 12 jaar of jonger). Onderzoek naar de farmacokinetische data van de primaire metaboliet van midazolam (α-hydroxymidazolam) toonde aan dat de grootste omzetting werd gezien in de jongste kinderen en derhalve de hoogste CYP3A4/5 activiteit.

Hoofstuk 8 beschrijft de resultaten van een farmacokinetische studie met midazolam en α-hydroxymidazolam na een intraveneuze toediening van 0.1 mg/kg aan 24 preterme pasgeborenen (zwangerschapsduur 26 - 34 weken, postnatale leeftijd 3 - 11 dagen).

Ondanks het feit dat er geen significante associaties werden gevonden tussen leeftijd (zwangerschapsduur, postnatale leeftijd of postconceptionele leeftijd) en de plasmaklaring van midazolam als gevolg van de grote interindividuele variabiliteit (ongeveer 10-voudig), was de gemiddelde waarde van de plasmaklaring voor preterme pasgeborenen significant lager dan de waarden die werden gezien bij voorafgaande studies verricht bij oudere kinderen en adolescenten (**Hoofdstuk 7**). Dit laat dus een duidelijke ontwikkelingsafhankelijke reductie in CYP3A4/5 activiteit zien. Onderzoek naar de omzetting van midazolam naar de grootste metaboliet (α-hydroxymidazolam) bevestigde dit. Een toevallige, maar belangrijke bevinding tijdens dit onderzoek was het effect van gelijktijdige toediening van indomethacine (voor de behandeling van een open ductus Botalli) op de dispositie van midazolam. Dit toonde aan dat er een duidelijke interactie is tussen enerzijds ontwikkeling en anderzijds behandeling van ziekteprocessen op de expressie van CYP3A4/5 activiteit gedurende de eerste twee levensweken.

Het ontwikkelen van een selectief modelmedicijn voor CYP3A4
Eerder onderzoek waarbij modelmedicijnen werden gebruikt om de activiteit van CYP3A4 te meten lieten tegenvallende resultaten zien. In sommige gevallen waren de gebruikte modelmedicijnen polyfunctionele substraten voor cytochroom P450 (b.v. dapsone, dextromethorphan) en derhalve niet selectief bruikbaar voor het meten van CYP3A4 activiteit. Bij gebruik van erythromycine bleek dat dit middel actief ook door P-glycoproteine werd getransporteerd met als gevolg dat de dispositie van erythromycine niet de activiteit van slechts een enkel enzym weerspiegelde. Midazolam, een stof die in het kader van dit proefschrift werd bestudeerd (**Hoofdstukken 7 en 8**) geeft een nauwkeurige schatting van de totale CYP3A activiteit omdat het een substraat is voor zowel CYP3A4 als CYP3A5. CYP3A5 komt in ruime mate tot expressie in de menselijke lever waarbij er een duidelijke relatie bestaat met het ras. Er was derhalve geen selectief modelmedicijn beschikbaar om CYP3A4 activiteit te meten wat ertoe leidde dat het onmogelijk was de rol van ontwikkeling op dit specifieke iso-enzym *in vivo* te meten.

Hoofdstuk 9 beschrijft de resultaten van de eerste van een serie studies die werden verricht om het gebruik van cisapride als een selectief modelmedicijn om CYP3A4 activiteit te meten te valideren. Phenotypering werd verricht met behulp van gefenotypeerde levermicrosomen en microsomen bereid uit door baculovirus geïnfecteerde insect cellen die 10 verschillende menselijke cytochroom P450s tot

expressie brengen waaronder CYP3A4, 3A5 en de foetale vorm CYP3A7. De omzetting van cisapride naar norcisapride en twee andere belangrijke metabolieten, 4-fluoro-2-hydroxy cisapride (4F) en 3-fluoro-4-hydroxycisapride (3F) werd onderzocht door gebruik te maken van een nieuwe HPLC/MS methode. Tevens werden een aantal studies verricht die gebruik maakten van zeer goed gekarakteriseerde farmacologische remmers van geselecteerde P450 cytochromen. Gezamenlijk toonden deze studies aan dat de vorming van norcisapride en de twee andere metabolieten overeenkwamen met Michaelis-Menten kinetiek betreffende een enkel enzym. Onderzoek met enzymen die c-DNA tot expressie brengen toonde aan dat CYP3A4 de omzetting van cisapride (in concentraties gelijk aan welke worden gezien in menselijk plasma tijdens behandeling met cisapride) tot norcisapride en 4F meer dan 100 maal sneller gaat dan via niet-CYP3A enzymen, 50 maal sneller dan via CYP3A5 en 100 maal sneller dan via CYP3A7. Bij cisapride concentraties in de therapeutische range is CYP3A4 verreweg het belangrijkste iso-enzym voor het omzetten van cisapride met een slechts zeer marginale rol voor andere P450 cytochromen. Deze *in vitro* studies toonden dus aan dat cisapride een belangrijke kandidaat is om dienst te doen als modelmedicijn om CYP3A4 activiteit te meten.

De volgende stappen in het evalueren van cisapride als een modelmedicijn was om na te gaan of dit geneesmiddel ook door P-glycoproteine werd getransporteerd en te onderzoeken of geselecteerde farmacokinetische parameters (plasmaklaring, AUC) *in vivo* overeenkomen met dit CYP3A4 modelmedicijn.

In **hoofdstuk 10** worden de resultaten van een *in vitro* en *in vivo* studie met cisapride beschreven. Bidirectionele transportassays werden uitgevoerd met LLC-PK1 cellen en de L-MDR1 cellijn om vast te stellen of cisapride een substraat voor P-glycoproteine was. Cisapride was weliswaar geen substraat maar remde wel de P-gp activiteit met een Ki van 16.1 μM. Uit deze studie bleek dat, gebaseerd op de *in vitro* gegevens, cisapride de juiste selectiviteit bezat om bestudeerd te worden als een selectief modelmedicijn voor CYP3A4. Er werd derhalve een *in vivo* studie uitgevoerd bij 17 gezonde volwassenen die, op verschillende momenten, een intraveneuze (0.025 mg/kg) dosis midazolam, een orale (0.15 mg/kg) dosis midazolam, en een orale (0.07 mg/kg) dosis cisapride kregen toegediend. Na iedere toediening werd een farmacokinetische studie verricht. Er bleek een statistisch significante correlatie te zijn tussen de klaring van cisapride na orale toediening en de klaring van cisapride na zowel orale als intraveneuze toediening. Hiermee werd dus

de overeenkomst tussen deze CYP3A4 modelmedicijnen aangetoond. De plasmaklaring en AUC van cisapride kon op een nauwkeurige en betrouwbare wijze worden vastgesteld door het bepalen van een enkelvoudige 8 of 12-uurs plasma concentratie. De resultaten van deze voorlopige studies met cisapride (**hoofdstuk 9 en 10**) gekoppeld aan de wereldwijde klinische ervaring met dit geneesmiddel bij neonaten en kinderen suggereert dat cisapride gebruikt kan worden om de totale hoeveelheid CYP3A4 activiteit te bepalen en de ware rol van de ontwikkeling op dit iso-enzym vast te stellen.

De resultaten van een farmacokinetisch onderzoek met cisapride dat werd ontworpen om de ontwikkeling van CYP3A4 *in vivo* vast te stellen worden gepresenteerd in ***Hoofdstuk 11***. Tijdens dit onderzoek kregen 35 zuigelingen met een postconceptionele leeftijd van 30 - 51 weken een eenmalige orale dosering (0.2 mg/kg) cisapride toegediend op de postnatale leeftijd van 4 - 87 dagen. Plasma concentraties van zowel cisapride als norcisapride werd bepaald en gebruikt om farmacokinetische parameters te bepalen. Tevens werd de renale klaring van cisapride vastgesteld. Bij alle kinderen was de niet-renale klaring verantwoordelijk voor de plasmaklaring van cisapride. Onderverdeling van de onderzoeksgroep toonde aan dat de plasmaklaring van het geneesmiddel (0.45 l/uur/kg) bij de jonge, meest immature kinderen (postconceptionele leeftijd 28 - 36 weken) ongeveer de helft was van de klaring die gezien werd bij kinderen met een postconceptionele leeftijd van meer dan 36 weken. Tevens werd een positieve lineaire correlatie gevonden tussen de eliminatiesnelheidsconstante voor cisapride en de postconceptionele leeftijd. Onderzoek van de ratio's betreffende de AUC van norcisapride:cisapride leverde dezelfde mate van variabiliteit op als eerder gerapporteerd voor de normale interindividuele variabiliteit voor CYP3A4 bij volwassenen (ongeveer 25-voudig). Onderzoek van de farmacokinetische data van cisapride als een functie van de leeftijd liet een dramatische toename van CYP3A4 activiteit in de eerste maanden van het leven zien. Hierdoor was een *in vivo* bewijs geleverd voor van de rol van ontogenie op de activiteit van dit specifieke cytochroom P450 isoform.

Het integreren van farmacogenetica in studies om de rol van leeftijd op geneesmiddel-dispositie na te gaan

Hoofdstuk 12 geeft een overzicht en een commentaar betreffende de behoefte en uitdagingen om farmacogenomie in de klinische kindergeneeskunde te introduceren. De toepasbaarheid van farmacogenetica en farmacogenomie wordt bediscussieerd in een

ontwikkelingsperspectief evenals de uitdagingen die specifiek horen bij kindergeneeskundige ziektebeelden (inclusief geneesmiddel-geinduceerde condities) en de ontwikkeling van relevante modelsystemen. Aanvullend worden speciale ethische en wetenschappelijke overwegingen beschreven die enerzijds, indien slecht uitgevoerd, de toepassing van de farmacogenomie kunnen beperken en anderzijds, indien correct uitgevoerd, het onderzoek van geneesmiddelen bij kinderen kunnen versterken. Dat het geneesmiddelenonderzoek bij kinderen versterkt kan worden, wordt bewezen door **hoofdstukken 13 en 14** waarin relevante farmacogenetica geïntegreerd wordt in twee klinische trials uitgevoerd bij kinderen als onderdeel van een officieel geneesmiddelontwikkelingsprogramma.

Omeprazol, een protonpomp remmer en substraat voor het enzym CYP2C19, dat zich kenmerkt door polymorfe expressie, wordt routinematig gebruikt bij zuigelingen, peuters, kleuters, kinderen en adolescenten in zowel de Verenigde Staten, Canada en Europa als behandeling van gastro-oesofageale reflux en een scala aan andere ziektebeelden waarvoor controle van de pH van de maaginhoud noodzakelijk is.

Hoofdstuk 13 beschrijft de resultaten van een farmacokinetische studie met dit geneesmiddel uitgevoerd bij 37 kinderen en adolescenten, variërend in leeftijd van 2-16 jaar, na de toediening van een eenmalige orale dosis (10 of 20 mg) van het geneesmiddel. Omdat de farmacokinetiek en farmacodynamiek van omeprazol bij volwassenen sterk worden beïnvloed door het CYP2C19 genotype, werd in deze studie CYP2C19 genotypering (een maat voor het CYP2C19 fenotype) toegevoegd aan het studieprotocol om 1). eventuele "uitschieters" van de farmacokinetische data te kunnen beschrijven en 2). na te gaan hoeveel van de variabiliteit in de dispositie van omeprazol te verklaren valt door leeftijd versus farmacogenetische (CYP2C19 genotype) determinanten. In tegenstelling tot eerdere aannames werd door ons geen leeftijdafhankelijkheid gevonden wat betreft de dispositie van het geneesmiddel bij kinderen variërend tussen 2 en 16 jaar. De mate van systemische blootstelling geassocieerd met inname van 10 - 20 mg omeprazol bij kinderen was gelijk aan wat gezien wordt bij volwassenen die therapeutische doseringen nemen. Slechts 1 kind met een langzaam CYP2C19 genotype werd geïdentificeerd in dit studie cohort en dit kind toonde, zoals te verwachten was, significant hogere AUC en piek plasmaconcentraties dan de kinderen uit de studie met een genotype passend bij snelle omzetting van omeprazol. Bestudering van de

farmacokinetische data van omeprazol toonde aanzienlijke variatie van zowel AUC als plasmaklaring waardoor onderzoek gestart werd naar een mogelijk gen-dosis effect. Echter, vergelijking van gemiddelde AUC waarden genormaliseerd voor dosis (per 1mg/kg) van individuen met 1 versus 2 functionele CYP2C19 allelen liet geen statistisch significante verschillen zien.

Chapter 14 beschrijft de resultaten van een klinisch pediatrische farmacokinetiek studie met tramadol, een niet-morfineachtig analgeticum, dat een substraat is voor CYP2D6, een enzym met polymorfe expressie. Deze studie onderzocht de plasma verdeling van tramadol en haar metabolieten (M1 en M2) bij 26 kinderen (7 - 16 jaar) die een eenmalige orale dosis kregen toegediend. Bij een subgroep van dit studie cohort (n = 13) werd CYP2D6 genotypering verricht en tevens bepaling van het fenotype door bepaling van dextromethorphan en de primaire metabolieten (dextrorphan, 3-methoxymorphinan en 3-hydroxymorphinan) in de urine na een eenmalige orale dosis dextromethorphan. In deze studie werd geen leeftijdsafhankelijkheid van de farmacokinetische parameters van tramadol gezien. Echter, er werd een lichte correlatie gezien tussen de tramadol/M1 plasma concentratie en de dextromethorphan/dextrorphan urine ratio. Een veel sterkere relatie werd gevonden bij kinderen met twee, in tegenstelling tot één functionele allel, indien de kinderen gescheiden werden op basis van het aantal functionele CYP2D6 allelen. Nadere evaluatie van de data suggereert dat vorming van M1 (CYP2D6 gemedieerd) verminderd is en de vorming van M2 (niet door CYP2D6 gemedieerd) toegenomen is in individuen met één versus twee functionele allelen. Er was dus een potentieel gen-dosis effect voor CYP2D6 gemedieerde geneesmiddelomzetting aanwezig.

15.5 Toekomstperspectief

Ondanks de enorme vooruitgang die geboekt is in het vakgebied van de klinische kinderfarmacologie gedurende de laatste tien jaren blijven er nog grote leemten in onze kennis met betrekking tot onze mogelijkheden om volledig en in vele gevallen op een nauwkeurige wijze de rol van leeftijd op de activiteit van enzymen die van belang zijn voor het metabolisme van geneesmiddelen te karakteriseren. Farmacokinetische studies van therapeutisch gebruikte geneesmiddelen zijn beperkt bruikbaar voor dit doel omdat het merendeel van de geneesmiddelen gemetaboliseerd wordt door een groep van

verschillende enzymen (zogenaamde polyfunctionele substraten). Als gevolg hiervan is het beeld van ontogenie dat deze studies ons geven beperkt en niet compleet.
Het post-genoom tijdperk biedt nog niet eerder bestaande mogelijkheden om belangrijke bijdragen te leveren aan de klinische kindergeneeskunde door het op verstandige en doelgerichte wijze inbouwen van farmacogenetische en/of farmacogenomische instrumenten in klinische trials die ontworpen zijn om de dispositie, actie en effectiviteit van geneesmiddelen te bestuderen. Uit dit proefschrift blijkt dat de kwaliteit van een farmacokinetische studie sterk kan verbeteren door verklaringen te geven van een extreem afwijkend fenotype en tevens de mogelijkheid om bij onderzoek dat ontworpen is voor het vaststellen van de rol van ontogenie op geneesmiddeldispositie, onderscheid te maken tussen enerzijds de rol van ontwikkeling en anderzijds de normale, te verwachten interindividuele variabiliteit (in de activiteit van geneesmiddelmetabolisme) dat in grote mate onderhavig is aan genetische controle. Het includeren van farmacogenetica (of farmacogenomie) in een klinische farmacokinetische studie heeft een duidelijke rol om zowel de interpretatie als het uiteindelijke klinische nut te verbeteren.
Recent zijn twee nieuwe grensgebieden "ontstaan" tussen de ontogenie en de farmacogenetica. Het eerste gebied is het identificeren van "triggers" die leeftijdgebonden veranderingen in de activiteit van enzymen betrokken bij geneesmiddelmetabolisme veroorzaken. Dit maakt het noodzakelijk een gedetailleerde studie te verrichten van gebeurtenissen die genexpressie controleren zoals xenobiotische interacties met kern receptoren met polymorfe expressie, repressie en/of de-repressie van post-receptor onderdelen van de promotor en/of regulatoire delen van kandidaat genen. Het tweede gebied betreft het ontdekken van farmacogenetische determinanten voor geneesmiddeleffect. Dit maakt het noodzakelijk de rol van specifieke genen, SNIPs of haplotypes voor enerzijds de etiologie van ziekte en anderzijds de uitkomst van behandelingstrategieën, vast te stellen. Tot slot, om er voor te zorgen dat deze nieuwe inzichten in het tijdperk van het bekend worden van het menselijk genoom ook daadwerkelijk vertaald gaan worden in ontdekkingen die op een gunstige wijze de zorg en behandeling van kinderen beïnvloeden is het noodzakelijk dat de maatschappij (zowel wetenschappers als het gewone publiek) de vele mogelijkheden die deze nieuwe kennis ons brengt omarmt. Iedere poging dient ondernomen te worden om deze kennis te integreren in de ontwikkeling van geneesmiddelen die essentieel zijn om de gezondheid van kinderen te verbeteren.

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Curriculum vitae

Gregory Lee Kearns was born on June 3rd, 1954 in St. Louis, Missouri. He graduated from High School (New Athens Community High School, New Athens, Illinois) in 1972 and in that same year, began a course of study at the St. Louis College of Pharmacy from which he graduated (cum laude) in 1977 with a Bachelor of Science degree in Pharmacy. In July of 1977, he entered graduate school at the University of Cincinnati and was privileged to study clinical pharmacokinetics in the laboratory of prof. dr. Wolfgang A. Ritschel. In 1979, he was awarded a Doctor of Pharmacy degree from the University of Cincinnati Division of Graduate Education and Research. From July of 1977 through June of 1979, he also completed a residency program in Pediatric Pharmacotherapy at the Children's Hospital Medical Center in Cincinnati, Ohio. In July of 1979, Dr. Kearns began a postdoctoral fellowship in Clinical Pharmacology at the Louisiana State University School of Medicine in Shreveport, Louisiana. From 1979 through February of 1983, he studied under the able mentorship of prof. dr. John T. Wilson and prof. dr. R. Don Brown where, in addition to completing additional graduate coursework in Pharmacology, he conducted research in pharmacokinetics (both clinical and experimental), pharmacodynamics and developmental pharmacology. In March of 1983, he joined the faculties of Pharmacy and Medicine at the University of Arkansas for Medical Sciences in Little Rock, Arkansas as an Assistant Professor of Pharmaceutics and Pediatrics, where he remained through January of 1996. During his tenure at the University of Arkansas for Medical Sciences, Dr. Kearns was promoted to the rank of Associate Professor (with tenure) in 1989 and to Professor in July of 1994. In addition to conducting research in pediatric clinical pharmacology, he worked under the direction of the late prof. dr. Donald E. Hill in establishing the piglet as a model to study drug disposition in developing humans and also, began a professional collaboration with prof. dr. John N. van den Anker from the Sophia Children's Hospital in Rotterdam. In July of 1988, Dr. Kearns established the Section of Pediatric Clinical Pharmacology and Toxicology at the Arkansas Children's Hospital where he served as Chief until January of 1996. In the eight-year history of this program, he served as the primary mentor for two postdoctoral fellows; James D. Marshall, M.D. and Laura P. James, M.D. In January of 1996, Dr. Kearns left the University of Arkansas for Medical Sciences to accept an appointment as a Professor in the Departments of Pharmacology and Pediatrics at the University of Missouri – Kansas City, and to serve as Director of a new Division of Pediatric Pharmacology and Medical Toxicology at the Children's Mercy Hospitals and Clinics in Kansas City, Missouri. He was honored at this time by being named the recipient of the

Marion Merrell Dow / Missouri Endowed Chair in Pediatric Pharmacology, a position that he currently holds. From 1996 to the present, Dr. Kearns has served on numerous occasions as a Visiting Professor and Lecturer in the Department of Pediatrics of the Erasmus University and Sophia Children's Hospital in Rotterdam. He was also privileged to serve as a member of the Erasmus University Ph.D. committee for prof. dr. John N. van den Anker (October 1995) and dr. Saskia N. de Wildt (September 2000). During the past seven years, he has enjoyed a close, extensive professional research collaboration with prof. dr. John N. van den Anker; many of the fruits of which formed the basis of and are described in this Ph.D. thesis.