Pharmacologic Modulation of Topoisomerase I Inhibitors

Diederik Kehrer

Cover design: D.F.S. Kehrer en H.S. Kehrer

Lay-out: P.J. Bos, Dept. of Medical Oncology
Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam

Printed by: Optima Grafische Communicatie

ISBN: 90-9015207-5

Publication of this thesis was financially supported by:
Gilead Science Inc., Amgen, Astra-Zeneca, Aventis, Bristol Meijers Squibb, Glaxo Wellcome, Novartis, Ortho Biotech, Pierre Fabre, Roche.

Pharmacologic Modulation of Topoisomerase I Inhibitors

Farmacologische Modulatie van Topoisomerase I Remmers

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof.dr.ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties

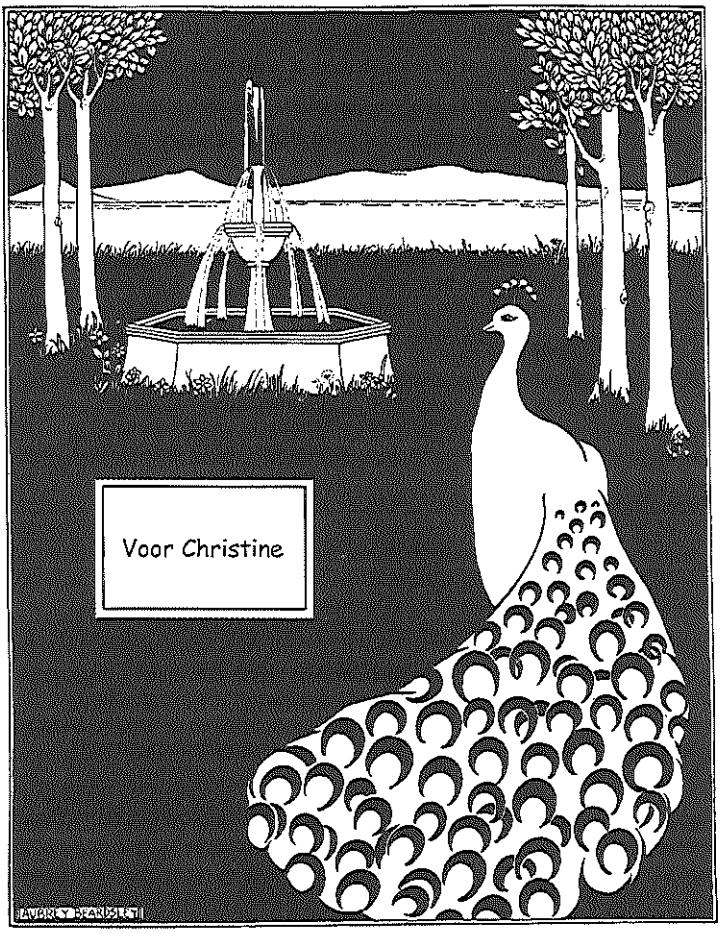
De openbare verdediging zal plaatsvinden
op vrijdag 16 november 2001 om 13.30 uur

door

Diederik Frans Sebastiaan Kehrer
geboren te Voorthuizen

PROMOTIECOMMISSIE

Promotor:	Prof.dr. J. Verweij
Overige leden:	Prof.dr. G. Stoter
	Prof.dr. J.H.M. Schellens
	Prof. J.H.P. Wilson
Copromotor:	Dr. A. Sparreboom
	Dr. M.J.A. de Jonge


Voor Christine

Contents

Introduction to the Thesis

Introduction

Camptothecin, a plant alkaloid isolated from *Camptotheca acuminata,* was identified in the late 1950's. Due to severe and unpredictable toxic side effects in early clinical studies, the clinical development of this drug was halted in the 1970's. In the early 1980's several important events occurred that resulted in renewed interest in this agent. The molecular target of camptothecin, the nuclear enzyme topoisomerase I, was identified. This topoisomerase I was described as an enzyme involved in transient scission and relegation of DNA during the replication and transcription phases. Binding of camptothecin to the topoisomerase I-DNA complex (cleavable complex) and interference with the relegation step of this process was recognized as the primary mechanism of action of camptothecin, finally leading to a double stranded DNA break and, ultimately, cell death. At the same time, it was shown that the failures encountered in the clinical development of camptothecin were related, at least partially, to the drug's poor water solubility, which necessitated pharmaceutical formulation in alkaline solutions for i.v. administration. This not only led to chemical modification of the original structure into an entity lacking antitumor activity, but also induced profound alterations in the toxicological behavior of the agent.

These two key findings then boosted drug-research efforts aimed at identifying and developing new analogues with improved water solubility while maintaining the unique mechanism of action. Some of these agents are currently in clinical development, while others, among which irinotecan (CPT-11), is now registered for use in colorectal cancer. CPT-11 underwent extensive clinical evaluation in phase II and III trials and data suggested that the drug is also active in various other tumor types in addition to the indication mentioned. The main side effects of this drug are bone marrow suppression and late onset diarrhea.

CPT-11 is a prodrug with limited antitumor activity. CPT-11 is mainly metabolized by two metabolic pathways. One pathway is the hydrolyzation by a carboxylesterase to form the active metabolite SN-38, which is 100 to 1000-fold more active than the parent compound. SN-38 in its turn can be metabolized further by UDP glucuronosyltransferase 1A1 to form an inactive β-glucuronide derivative (SN-38-G), which in turn is excreted in the bile and subsequently the bowel system. The other pathway is the oxidative elimination by the cytochrome P-450 system to form the inactive metabolites APC and NPC. The complex metabolism in combination with the earlier mentioned clinical toxicity of CPT-11, raises questions on probable pharmacological ways to modulate these pathways and toxicity.

In *in vitro* studies, topoisomerase I inhibitors showed more pronounced antitumor efficacy with protracted exposure to low concentrations. Also in animal models, low dose prolonged exposure resulted in less toxicity. Clinical studies have focused on low-dose exposure to topoisomerase I inhibitors. Based on this knowledge, considerable effort has recently been put in the development of alternative pharmaceutical vehicles that would allow prolonged systemic exposure. Among various approaches, liposomal encapsulation of an intravenously

administered drug would greatly prolong the exposure. Liposomal encapsulated anticancer drugs have been studied extensively both in the laboratory and in the clinic, with reports of prolonged plasma exposure, improved tumor delivery, decreased systemic toxicity and increased efficacy for a variety of cytotoxic drugs. In addition, the topoisomerase I inhibitor lurtotecan, in analogy to other topoisomerase I inhibitors, is significantly influenced by a chemical pH-dependent hydrolysis of the active lactone form to the ring-opened biologically inactive carboxylate form. Inside liposomes the pH can be modified. So encapsulating topoisomerase I inhibitors in liposomes can be beneficial both in the sense of prolonged exposure as to maintain the drug in the active lactone form.

Besides the earlier mentioned important recognition of the workings mechanism of topoisomerase I inhibitors, improved understanding of the signal transduction pathways has resulted in identification of other potential therapeutic targets, among which farnesyl transferase inhibitors. Farnesyl transferase inhibitors were specifically developed to decrease Ras processing and subsequently downregulate transduction of proliferative signals. Ras mutations have been demonstrated in the majority of colon cancers. Seen the distinctly different mode of action of farnesyl transferase inhibitors and topoisomerase I inhibitors, and the need of more efficacious treatment for (colon) cancer, a strong rationale exists for investigating a CPT-11 based regimen in combination with farnesyl transferase inhibitors.
This thesis, "pharmacological modulation of topoisomerase I inhibitors", includes studies on the liposomal encapsulated topoisomerase I inhibitor lurtotecan, studies on pharmacological modulation of CPT-11 with common drugs and combination therapy of CPT-11 with the farnesyl transferase inhibitor R115777.

Chapter 1

Modulation of Camptothecin Analogues in the Treatment of Cancer: a Review

D.F.S. Kehrer, O. Soepenberg, W.J. Loos, J. Verweij, A. Sparreboom

Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam, The Netherlands

Anti-Cancer Drugs 12: 89-105, 2001

Abstract

The topoisomerase I inhibitors reviewed in this paper are all semisynthetic analogues of camptothecin. Modulation of this intranuclear enzyme translates clinically in antitumor activity against a broad spectrum of tumors, and is therefore subject of numerous investigations. We present preclinical and clinical data on camptothecin analogues that are already being used in clinical practice [i.e., topotecan and irinotecan (CPT-11)] or are currently in clinical development (e.g., 9-aminocamptothecin, 9-nitrocamptotecin, lurtotecan, DX 8951f and BN 80915), as well as drugs that are still only developed in a preclinical setting (Silatecans, polymer-bound derivates). A variety of different strategies is being used to modulate the systemic delivery of this class of agents, frequently in order to increase antitumor activity and/or reduce experienced side effects. Three principal approaches are being discussed, including (i) pharmaceutical modulation of formulation vehicles, structural alterations and the search for more water soluble prodrugs, (ii) modulation of routes of administration and considerations on infusion duration, and (iii) both pharmacodynamic and pharmacokinetic biomodulation.

Introduction

Camptothecin (CPT), a plant alkaloid isolated from *Camptotheca acuminata*, was first identified in the late 1950's [1]. Due to severe and unpredictable toxic side effects in early clinical studies, including myelosuppression, severe diarrhea and hemorrhagic cystitis, the clinical development of CPT was halted in the 1970's [2-5]. In the early 1980's several important events occurred that resulted in renewed interest in this agent; (i) the molecular target of CPT, *viz.* the nuclear enzyme topoisomerase I, was identified. This topoisomerase I was described as an enzyme involved in transient scission and relegation of DNA during the replication and transcription phases. Binding of CPT to the topoisomerase I-DNA complex (cleavable complex) and interference with the relegation step of this process was recognized as the primary mechanism of action of CPT, finally leading to a double stranded DNA break and, ultimately, cell death [6-8]. Subsequent investigations indicated overexpression of this topoisomerase I enzyme in various types of solid tumors, including ovarian and colon cancer [9,10]. (ii) At the same time, it was shown that the failures encountered in the clinical development of CPT were related, at least partially, to the drug's poor water solubility, which necessitated pharmaceutical formulation in alkaline solutions for i.v. administration. This not only led to chemical modification of the original structure into an entity lacking antitumor activity, but also induced profound alterations in the toxicological behavior of the agent [11]. These two key findings then boosted drug-research efforts aimed at identifying and developing new [(semi-)synthetic] analogues with improved aqueous solubility while maintaining CPT's unique mechanism of action. Some of these agents are currently in clinical

development, whilst irinotecan (CPT-11) and topotecan are now registered for use in colorectal cancer [12], and ovarian and lung cancer [13], respectively. Both topotecan and CPT-11 underwent extensive clinical evaluation in phase II and III trials and data suggested that both drugs are also active in various other tumor types in addition to the indications mentioned [14,15].

Unlike most other CPT analogues, CPT-11 is a prodrug with very little inherent antitumor activity. To form the active metabolite SN-38, which is 100 to 1000-fold more active than the parent compound [16], CPT-11 is hydrolyzed by a carboxylesterase [17]. SN-38 in its turn can be metabolized further by UDP glucuronosyltransferase 1A1 to form an inactive β-glucuronide derivative (SN-38-G) [18]. In *in vitro* studies, topoisomerase I inhibitors showed more pronounced antitumor efficacy with protracted exposure to low concentrations. Also in animal models, low dose prolonged exposure resulted in less toxicity [19-23]. It should be noted that for several reasons, including species differences in drug disposition and tolerability as well as intrinsic differences in tumor sensitivity, *in vitro* and animal models have been shown to be poor predictors of clinical efficacy and toxicity. Nonetheless, most clinical studies have focussed on low dose exposure to topoisomerase I inhibitors in cancer patients [24-29]. Meanwhile, numerous researchers are unravelling the clinical pharmaco-dynamics and pharmacokinetics of the different analogues. This has lead to an explosion in publications on this subject. The present review is focussed on chemical and pharmacologic aspects of CPT analogue development, with special emphasis on the choice of routes of delivery and on intrinsic differences in toxicity profiles of the various analogues and possible ways to modulate these either pharmacodynamically or -kinetically.

Chemical Properties

Structure-Activity Relationships

Most of the currently known CPT analogues share a basic 5-ring structure with a chiral center located at C20 in the terminal E-ring. Extensive studies on the synthesis of CPT analogues and the development of structure-activity relationships have been carried out over the last several years, and some important general relationships have emerged [30]. While these relationships will clearly be refined in the years to come, current knowledge is potentially adequate for the design of improved analogues of CPT. This current knowledge is here summarized in Figure 1 rather than being described exhaustively. Structure-activity studies have also shown a close correlation between the ability to inhibit topoisomerase I and overall cytotoxic potency [31]. For the purpose of this review, a number of regions in the CPT structure is particularly relevant:

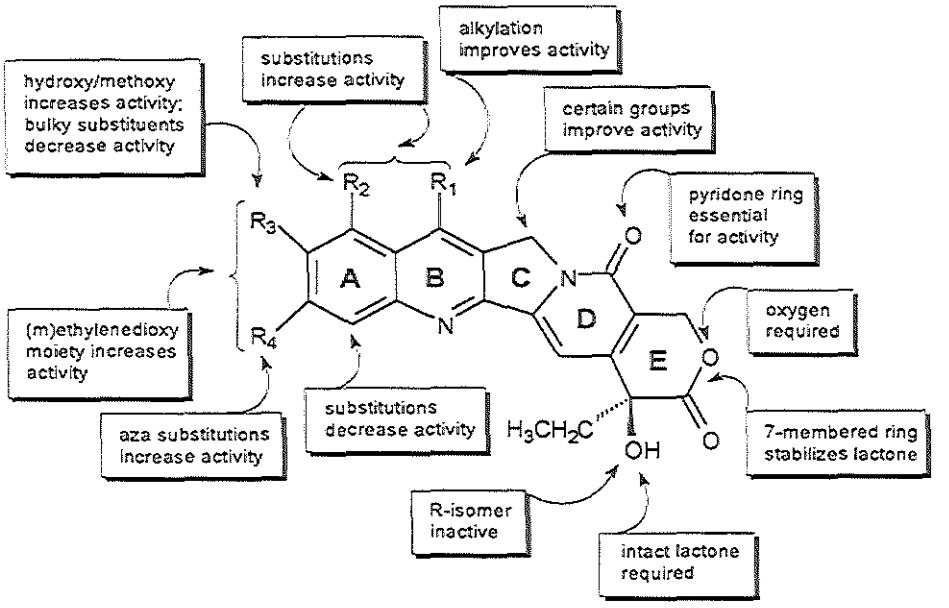


Figure 1. *Structure-activity relationships of camptothecins*

(i) It has been shown that the topoisomerase I inhibitory activity of these agents is stereospecific, with the naturally occurring (S)-isomer being many-fold more potent that the (R)-isomer [7-9].

(ii) In general, substitutions at C7, C9 and C10 tend to increase topoisomerase I inhibition and sometimes increase water solubility, whereas substitutions at C12 decrease antitumor activity [16].

(iii) Similarly, the formation of certain additional ring structures, e.g. between C7-C9 or C10-C11 increases activity [32-35].

(iv) One of the principal chemical features of this class of agents is the presence of a lactone functionality in the E-ring, which is not only essential for antitumor activity, but it also confers a degree of instability to these agents in aqueous solutions [36]. All known camptothecins can undergo a pH-dependent reversible interconversion between this lactone form and a ring-opened carboxylate (or hydroxy acid) form (Figure 2), of which only the lactone form is able to diffuse across cell membranes, and exert the characteristic topoisomerase I inhibitory activity. At neutral or physiologic pH, the equilibrium between the

two species favors the carboxylate form for all the camptothecins. As outlined, an understanding of this hydrolysis reaction helps to explain several observations in the early development of these agents. Because CPT was administered as the more water-soluble sodium salt, patients were exposed to high concentrations of the relatively inactive carboxylate species, whereas large amounts of drug were excreted in urine, where the low pH favored closure of the lactone ring with resulting hemorrhagic cystitis [2,4]. The equilibrium between the lactone moiety ring and the carboxylate form of the camptothecins is not solely dependent on the pH, but also on the presence of specific binding proteins in the biological matrix, most notably human serum albumin (HSA). Following establishment of equilibration at 37°C in phosphate buffered saline (PBS), equal amounts of the various CPT analogues are present in the pharmacologically active lactone form, with values of 17, 19, 15, 13 and 15% for CPT, 9-aminocamptothecin (9-AC), topotecan, CPT-11 and SN-38, respectively [37]. ddition of 40 mg/mL HSA shifts the equilibrium for CPT and 9-AC towards the carboxylate form, with approximately 1% present in the lactone form at equilibrium [37,38]. In contrast to HSA, addition of murine serum albumin to 9-AC leads to approximately 35% existing in the lactone form at equilibrium [38]. As opposed to CPT and 9-AC, HSA actually stabilizes the lactone moiety of CPT-11 and SN-38, with values of 30 and 39%, respectively, present in the lactone form at equilibrium, while almost no effect was seen for topotecan [37]. It has been proposed that the differences in the percentiles present in the lactone form at equilibrium is related to sterical considerations of the various substituents at the R_1 and R_2 positions (Figure 2A). For some of the more recently developed CPT analogues, the substituents cause sterical hindrance and prevent binding of the carboxylate forms to HSA, and so drive the equilibrium towards the lactone species.

Conventional Drug Formulations

The inherent instability of the lactone form and the inactivity of the carboxylate form have posed a specific challenge to the development of a suitable dosage form of CPT analogues. Since the lactone form is strongly favored by an acidic pH, as outlined above, the currently registered agents topotecan and CPT-11 have both been formulated in buffered dosage forms. Topotecan is available as a powder containing topotecan hydrochloride and tartaric acid, yielding an aqueous solution for infusion of pH 2.5-3.5 after reconstitution. This solution is stable for at least 12 hours at room temperature, whereas the dry powder is stable for at least 2 years at room temperature. The pharmaceutical dosage form of CPT-11 is similarly based on formulation of the hydrochloride form of the drug in an aqueous solution, containing a lactic acid-sodium hydroxide buffer system of pH 3.5-4.5. Current shelf-life studies have shown that the injection concentrate is stable for at least 3 years at room temperature when protected from light.

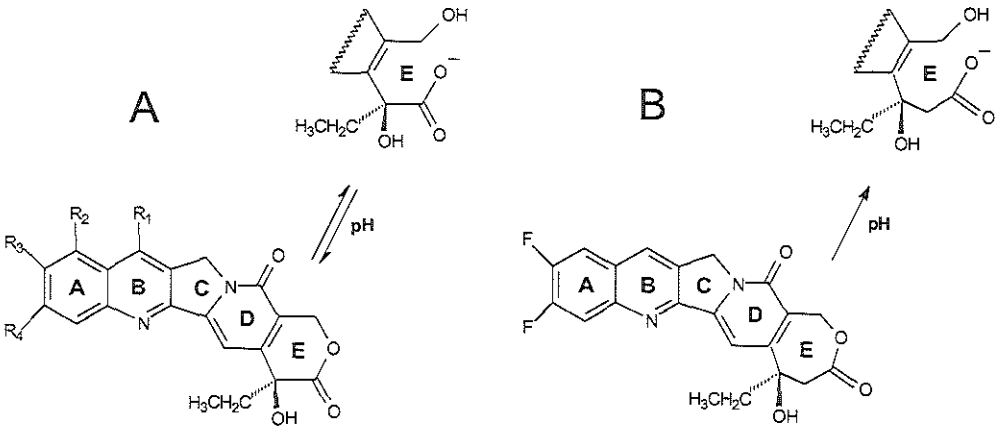


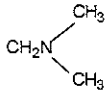
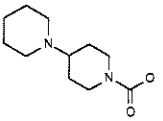
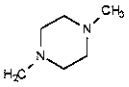
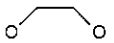
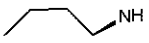
FIGURE A	R_1	R_2	R_3	R_4
CPT	H	H	H	H
9NC	H	NO_2	H	H
9AC	H	NH_2	H	H
TPT	H		H	H
CPT-11	CH_2CH_3	H		H
SN-38	CH_2CH_3	H	OH	H
LRT		H		
DX-8951			CH_3	F

Figure 2. *Chemical structures of camptothecin analogues*

Pharmaceutical Modulation

Alternative Formulations

In recent years, a variety of alternative pharmaceutical formulations have been or are currently being evaluated. Important properties of these alternatives will be that they allow drug doses to be delivered at levels (at least) similar to those achieved with the conventional formulations, and that the drugs should be stable for several hours in order to be handled in a clinical setting. Despite the tremendous efforts invested so far, only very few of the alternative have fulfilled the requirements to justify clinical testing. The rationale for re-formulation of CPT or its analogues has been either to stabilize the lactone moieties or to induce sustained release combined with specific tumor targeting of these agents. An example of the first approach has been the formulation of CPT, CPT-11 or 10-hydroxy-CPT in microspheres composed of poly-D,L-lactic acid or poly(D,L-lactic-co-glycolic acid) [39-42]. The influence of various encapsulation procedures on the release of these agents has been extensively examined, and has shown stabilization of the lactone form due to an acidic microclimate of the microspheres combined with enhanced pharmacokinetic characteristics in animal models [43]. Although from a theoretical perspective the modulation of CPT in this prolonged release system could be attractive in the way of reducing local toxicity and improving therapeutic efficacy, no phase I studies have been performed so far.

Alternatively, substantial progress has been made recently toward liposomal formulation of a number of important CPT analogues. Liposomes are microparticulate carriers that consist of one or more lipid bilayer membranes enclosing an internal aqueous phase. The most common constituents are synthetic or naturally occurring phospholipids and cholesterol. Although several reports address the considerations in choosing the specific liposome constituents and their physical properties (e.g. for CPT [21], topotecan [44,45] and CPT-11 [46,47]), relatively little information has been presented on toxicity profiles and antitumor activity. A recent study reported, however, that liposomal topotecan, encapsulated in sphingomyelin/cholesterol liposomes using an ionophore-generated proton gradient, was eliminated from the plasma much more slowly than the free drug, resulting in a 400-fold increase in systemic exposure [48]. The liposomal preparation also protected topotecan from lactonolysis and increased antitumor activity in both murine and human tumor models. Likewise, data have been generated demonstrating that unilamellar liposomal formulations of lurtotecan (NX 211 [49] and SPI-355 [50]) have significant therapeutic advantage over free drug and that the increased activity is consistent with increased systemic exposure and enhanced tumor-specific delivery of the drug. Based on these exciting data, several phase I clinical trials have been initiated with NX 211 given to cancer patients in either weekly or 3-weekly regimens, and preliminary findings corroborate the preclinical pharmacological profile of this agent [51].

CPT and a number of analogues, notably topotecan, have also been formulated in solid-lipid nanoparticles [composed of stearic acid, soybean lecithin and polyoxyethylene-polypropylene copolymer (Poloxamer 188)] [52] and dimethyl-beta-cyclodextrin to stabilize the lactone form [53]. Although these systems are promising sustained release and drug-targeting systems in various preclinical models, their clinical merit has not yet been evaluated.

Synthetic Derivatives

9-Aminocamptothecin

9-AC is a semisynthetic CPT derivative which showed outstanding preclinical activity against a wide spectrum of tumor types, including those of breast, colon, lung and prostate and melanoma [54]. In clinical trials, the drug has been very extensively studied using two different formulations based on the use of dimethylacetamide/polyethylene glycol 400 or a colloidal dispersion preparation, which enhances solubility and stability. Clinical phase I investigations have been conducted using a variety of i.v. administration schedules, including a 30-min infusion given daily for 5 days every 3 weeks, and more prolonged infusion schedules using 24-hour, 72-hour, 96-hour and 7-day or 21-day continuous dosing repeated every 4 weeks [55-61]. All of the studies report neutropenia as the dose-limiting toxicity, while thrombocytopenia is also frequent and sometimes severe. Gastrointestinal toxicity is the second most reported though not dose limiting. Other toxicities are considered mild to moderate. Numerous multi-institutional phase II studies have been conducted in several disease types, and overall, 9-AC shows only very modest single-agent activity and its further evaluation does not seem indicated [59,62-64]. It has been suggested that the lack of clinically relevant antitumor efficacy relates to substantial inactivation of the agent due to the unfavorable lactone/carboxylate ratio in patients [38].

Homocamptothecins

In the search for more stabile CPT analogues, the synthetic preparation of derivatives bearing a 7-membered E-ring, the so-called homocamptothecins has been described (Figuur 2) [65]. The lactone ring is stabilized by modification of the naturally occurring 6-membered α-hydroxylactone ring into a 7-membered β-hydroxylactone ring by insertion of a methylene spacer between the alcohol and the carboxyl moiety. The lead compound in this series, i.e. homocamptothecin (hCPT), has been shown to be more stable than CPT, remains a highly potent inhibitor of both cell growth with superior topoisomerase I inhibitory activity as compared to CPT [66-68] and, most interestingly, changes the sequence-specificity of the drug-induced DNA cleavage by topoisomerase I [69]. Indeed, in contrast to CPT which shows a rapid hydrolysis of the lactone moiety until a pH and protein dependent equilibrium has been reached, hCPT displays a slow and irreversible hydrolytic lactone ring-opening [70]. After a 3 h incubation of CPT and hCPT in human whole blood at 37°C, the fraction present

in the lactone form was 6% in the case of CPT and 80% in the case of hCPT. This remarkable difference is not only due to the slower ring opening of hCPT, but also to a higher affinity of hCPT for red blood cells [71]. Based upon this promising feature of hCPT, a series of derivatives of this agent was developed that combine enhanced plasma stability and potent topoisomerase I-mediated cytotoxicity. Various fluorinated analogues were subsequently found to have potent cytotoxic activity against several cell lines, including those overexpressing a functionally active P-glycoprotein [72]. Figure 2 shows the chemical structure of BN 80915, one of the most promising fluorinated hCPT analogues, which recently has entered clinical phase I testing.

Silatecans

Using a cascade radical annulation route to the CPT family, a novel series of CPT analogues, viz. 7-silylcamptothecins or silatecans, have been synthesized that exhibited potent inhibition of topoisomerase I, dramatically improved blood stability and sufficient lipophilicity to favor blood-brain barrier transit [73]. Preliminary evaluation in preclinical mouse models indicate that silatecans may hold significant promise for the treatment of high-grade gliomas and provide a rationale for proceeding with further (pre)clinical evaluation of their efficacy and safety versus commercially available CPT derivatives, including topotecan and CPT-11.

Hexacyclic Camptothecins

Two representative agents of the hexacyclic CPT analogues are currently under investigation. The first of these, lurtotecan (also known as GI147211 or GG211), is a water-soluble, totally synthetic derivative with a dioxalane moiety between C10 and C11 [74]. This agent has been evaluated clinically in various phase I and phase II trials using a 30-min i.v. infusion given daily for 5 consecutive days or as a 72-hour continuous i.v. infusion. [75-77]. The dose-limiting toxicity in both schedules was myelosuppression, including severe neutropenia and thrombocytopenia. Non-hematological toxicities were various and only mild to moderate. Because the oral bioavailability was highly variable and as low as 10% [78], alternative ways of drug administration are currently being developed, including a new liposomal formulation (NX 211; see above).

The second agent, DX-8951f or exatecan mesylate, is a new water-soluble, CPT analogue with an amino group at C1 and a fluorine at C5 [79]. DX-8951f showed superior and a broader spectrum of antitumor activity *in vitro* and *in vivo* in comparison with the other CPT analogues tested [80,81]. Recently, the results of a phase I evaluation of DX-8951f have become available, with the drug administered as a 30-min i.v. infusion given daily for 5 days every 3 weeks [82,83]. Brief, noncumulative neutropenia was the most common toxicity, and was seen consistently at doses greater than 0.5 mg/m^2/day. Other nonhematologic toxicities were mild to moderate in severity. Various other schedules, including a once every 3 weeks

regimen with 30-min i.v. infusions [84,85] and one based on continuous i.v. infusions of 5 to 21 days are presently under investigation [86]. The 30-min infusion regimen with daily administration for 5 consecutive days is now being tested in clinical phase II trials in various disease types, including non-small cell lung cancer [87], pancreatic cancer [88], ovarian cancer [89], and colorectal cancer [90].

Prodrugs

Because of the poor aqueous solubility of some CPT analogues, some major efforts have been put into the design and synthesis of more water-soluble prodrugs that could be more readily formulated than the parent drug. Many of the synthesized compounds have shown only marginal improvements in solubility or are too unstable to allow administration in a clinical setting. The instability of prodrug forms of, for example, CPT is particularly problematic, since the product of degradation (generally the parent drug) is insoluble and precipitates in aqueous solutions. Other synthetic approaches have produced fairly stable prodrugs, but the rate of active drug liberation proceeds at a too slow and variable rate. To date, two approaches in prodrug design have yielded agents that have progressed to clinical evaluation, *viz.* the 9-nitro derivative of CPT and polymer-coupled derivatives of CPT and DX-8951f.

C9-Substituted Derivatives

One of the most extensively studied agents of this class is 9-nitrocamptothecin (9-NC), which acts as a partial prodrug of 9-AC [91,92]. 9-NC has a nitro radical in the C9-position and is highly insoluble in water, and was initially identified as a precursor in the semi-synthetic production of 9-AC. Since nearly all human cells are able to convert 9-NC to 9-AC, including tumor cells, it has been proven difficult to identify whether 9-NC-mediated antitumor activity is directly associated with the parent drug alone or with 9-AC alone or the combination of both [93]. Preliminary evidence generated in clinical phase II trials suggest that 9-NC may be potentially be useful in the treatment of advanced pancreatic cancer and refractory ovarian cancer using a daily times 4 or 5 per week schedule with the drug given orally [94]. An extensive clinical phase II program is currently being conducted to test the efficacy of this agent against various malignant diseases [95,96].

Polymer-bound Derivatives

One of the possible ways to modulate anticancer agents is the use of its attachment to macromolecules because these high molecular weight prodrug carriers can lead to reductions in systemic toxicity, longer retention time within the body, alterations in biological distributions and possible improvements in therapeutic efficacy [97,98]. Its use is dependent on the concept of the enhanced permeability and retention (EPR) effect in solid tumors [99]. This EPR effect is based on four general characteristics of tumor tissues in comparison with

normal tissues: (i) hyper-vascularity, (ii) hyperpermeability, (iii) defective vascular architecture, and (iv) less efficacious drainage due to hypoplastic or minimally effective lymphatic system [99]. The pharmacokinetic characteristics of CPT were the starting-point to modulate its structure in this way. As indicated, CPT is highly insoluble in water, and by converting the C20-OH moiety into an ester coupled to an amino-acid spacer to allow better solubilization in aqueous environments, several macromolecular prodrugs of CPT have been generated in recent years [100]. Two of these, MAG-CPT and PEG-CPT have progressed to clinical evaluation [97,101]. The former consists of CPT covalently bound to a soluble polymer [N-(2-hydroxypropyl) methacrylamide (HPMA)] through a glycyl-aminohexanoyl-glycyl spacer, whereas the latter is a macromolecule derived through conjugation of chemically modified polyethyleneglycol with CPT at the C20-OH position. A variety of preclinical studies with MAG-CPT and PEG-CPT have shown stabilization and sustained release of CPT and also prolonged drug retention within experimental tumors [97,101]. Recently, the preliminary results of clinical phase I studies of PEG-CPT [102] and MAG-CPT (also referred to as PNU 166148) [103] have been reported, and indicate substantially prolonged exposure times to the active species as compared to administration schedules of the free drugs.

In an effort to prolong exposure times of DX-8951f to tumor tissues that might increase cytotoxic properties and antitumor efficacy, a prodrug has recently been generated by linking the agent to a biodegradable carrier via a peptide spacer (DE-310). Clinical evaluation of this prodrug to examine this hypothesis is currently being conducted.

Routes of Delivery

Intravenous Dosing and Considerations of Infusion Duration

I.v. administration of CPT analogues is currently the most commonly used route of delivery. The advantages of this route are many, including the fact that the total amount and duration of the administered drug can be controlled. As mentioned earlier, this has the advantage of controlled prolonged delivery of the drug. In this regard, most of the studies have been done with topotecan,[5] and several of the most promising regimens have been selected to enter phase II testing, viz. a daily times 5 every 3 weeks 30-min schedule [104,105], weekly or 3-weekly 24-hour infusion [106,107] and a 21-day continuous low dose infusion administered every 4 weeks [13,25,27,29,108]. Besides proposed schedule dependency on antitumor activity, toxicity also appears to vary considerably. Overall, the dose-limiting toxicity is myelosuppression, consisting primarily of neutropenia, whereas in some continuous infusion schedules thrombocytopenia was more pronounced [25,104]. Anemia and nonhematological side effects, including nausea, vomiting, diarrhea, fatigue, asthenia, alopecia and mucositis are common but usually mild, and do not seem to be schedule dependent. Most of the studies showed that prolonged exposure to i.v. administered

topotecan is feasible. Randomized comparison of the daily times 5 schedule every 3 weeks and 24-h infusion once a week for 4 weeks repeated every 6 weeks in patients with ovarian cancer suggests that the daily times five topotecan regimen was significantly superior with respect to response rate [100,110]. Randomized studies between the 5-day schedule and the 21-day continuous infusion schedule are not yet available [111].

Regarding the administration of CPT-11, different schedules are currently being used in Europe (350 mg/m^2 as a 90-min infusion once every 3 weeks) [112], the USA (125 mg/m^2 given weekly as a 90-min infusion for 4 or 6 weeks) [113] and Japan (100 mg/m^2 given weekly as a 30-min infusion) [114]. In all these dosing schedules, the total amount of CPT-11 that can be tolerated in any time period is similar [115]. Schedules with protracted infusions that have been investigated vary between 96 hours weekly to 14-days continuous infusion [116]. [26,117] The maximum tolerated dose in these studies (10-30 mg/m^2/day) is much lower then for the short duration schedules. Surprisingly, the AUC of the active metabolite SN-38 reaches comparable levels as reported for the short duration regimens, which is not completely understood. One possible explanation would be that the enhanced metabolism of CPT-11 relates to saturation of carboxylesterase-mediated conversion of CPT-11 with (high-dose) short infusion schedules. As for toxicity, myelosuppression and diarrhea are the dose-limiting events in all tested regimens. Like with topotecan, protracted low-dose schedules give more rise to thrombocytopenia [116] while in shorter schedules neutropenia is more prominent. The influence of infusion duration of antitumor activity has not yet been evaluated in a randomized setting.

Oral Dosing

As indicated, the high specificity in the mechanism of action of CPT analogues for the S-phase in the cell cycle has led to the recognition that the compounds may require prolonged exposure to maximize the fractional cell kill. In this regard, the availability of clinically useful oral formulations of currently available CPT analogues would provide increased convenience for the administration of chronic dosing regimens and the opportunity for cost-effective outpatient therapy [94]. Since most of the CPT analogues have relatively short terminal disposition half-lives, the use of protracted oral dosing is not necessarily the same as continuous i.v. infusion, although if the postulated concept of time over threshold concentration is a valid indication of both toxicity and efficacy, oral dosing can mimic continuous infusion regimens. Formal oral bioavailability studies have been conducted for several agents, and have yielded bioavailability values for topotecan of 30 to 44% [118-120], for 9-AC of ~50% depending on the formulation applied [121,122], and for lurtotecan of 11% [78]. Clinical data are not yet available for CPT-11, although murine data show an oral bioavailability of between 10 and 20%, depending on the dose administered [123].

The development toward suitable oral regimens for CPT analogues have to date been most extensively studied for topotecan using daily or bi-daily administration of 5-day [124],

10-day [125], or 21-day schedules [126]. A variety of clinical studies have shown that with an increase in prolonged topotecan administration by this route, a shift occurs in dose-limiting toxicity from hematological toxicity (mainly granulocytopenia) toward severe gastrointestinal side effects, most notably diarrhea [127]. These investigations further indicated that the schedule applied, rather than the applied systemic exposure per course seemed to be related to the type of experienced toxicity [127]. Based on these considerations, the daily time 5 schedule has been recommended for future studies. The need for further clinical development of the oral topotecan formulation became even more important in view of recent findings that the oral formulation has similar efficacy in the treatment of advanced ovarian and small-cell lung cancers as compared to the i.v. formulation, while less hematological toxicity was observed [128,129].

Based on theoretical considerations, including the fact that carboxylesterases are highly expressed in human liver and the gastrointestinal tract that could result in presystemic metabolism to SN-38 [130], it appears particularly attractive to deliver CPT-11 by the oral route. Indeed, the preliminary findings of substantially increased SN-38 to CPT-11 concentration ratios with oral CPT-11 administration as compared to i.v. administration seems to sustain this notion. [131,132] In addition, oral drug administration was associated with increased persistence of circulating levels of the lactone form of SN-38, which might be an additional advantage with potential pharmacodynamic importance [133]. The clinical utility of oral CPT-11 administration is currently under further investigation.

Local Drug Administration

Hepatic Arterial Dosing

The narrow therapeutic window of systemic administration of CPT and its analogues has prompted a search toward local drug administration, with the rationale to obtain selectively higher activity against locally confined tumors and/or lower systemic toxicity without loss of antitumor activity. The pharmacokinetic behavior of CPT-11 was recently compared during 5-day hepatic arterial and i.v. infusion in a group a cancer patients [134]. These findings indicated that arterial drug administration is leading to significantly higher conversion of CPT-11 into the active metabolite as compared to the i.v. administration, although the clinical relevance of this observation is, as yet, unknown [135]. In recent years, various agents have also been used for arterial embolization in an attempt to encapsulate the concomitantly administered chemotherapeutic agent, and, thereby, further enhance the local drug concentration. This concept has been tested with CPT-11 administered with hepatic arterial chemoembolization to patients with primary and metastatic hepatic malignancies [134]. Further studies are clearly required to confirm efficacy of this treatment and should aim at measuring systemic and tumor tissue concentrations.

Pulmonary Delivery

Liposomal aerosol formulations of CPT and 9-NC have recently been developed for nasal inhalation treatment of experimental lung tumors xenografted in nude mice. It was found that this preparation was strikingly effective in the treatment of these xenografts growing subcutaneously over the thorax at doses much lower than those traditionally used in preclinical models administered by other routes [136]. Interestingly, 9-NC aerosol therapy was also effective against established melanoma and osteosarcoma lung metastases [137]. Concurrent pharmacokinetic studies showed that this type of treatment results in a prompt pulmonary distribution at substantial levels that could not be achieved with conventional routes of delivery, including oral and intramuscular [138,139]. Overall, these data suggest that local delivery of CPT analogues to the respiratory tract by liposome aerosol treatment might offer advantages over existing methods in the treatment of some diseases.

Intraperitoneal Dosing

Intraperitoneal (i.p.) administration has been used as a strategy for increasing total drug delivery to ovarian cancers confined to the peritoneal cavity. The pharmacokinetic behavior of topotecan suggests that a substantial pharmacokinetic advantage might be obtained following i.p. injection. Indeed, recent clinical evidence suggests very slow peritoneal clearance of topotecan and high peritoneal:plasma concentration ratios of >10 after i.p. drug administration [140]. I.p. administration of CPT-11 has also been studied recently in animal models and showed some potential advantages over the i.v. route. It appeared that the therapy was more efficient with an increase in life span and was less toxic as compared to the i.v. route in mice bearing C26 colon tumors. In addition, substantially elevated AUCs of CPT-11 and SN-38 were found in the peritoneum, although plasma levels were comparable to i.v. dosing [140]. The clinical implications of these observations have not yet been evaluated.

Biomodulation

Pharmacodynamic Alterations

The use of biomodulators to increase the therapeutic index of chemotherapeutic treatment has made a significant impact on certain diseases [141]. A number of cancer chemotherapy biomodulators has been approved for clinical use in humans, and these agents can modulate anticancer drugs through either pharmacodynamic or –kinetic modulations. Among extensively studied biomodulating agents are the class of hematopoietic growth factors to decrease chemotherapy-induced neutropenia and anemia. Initial attempts to increase topotecan dose intensity with the use of granulocyte colony-stimulating factor (G-CSF) failed, since thrombocytopenia and fatigue rapidly emerged as dose-limiting effects with the daily time 5 schedule [142,143]. On the other hand, G-CSF administered after 5 daily infusions of topotecan permitted a 2.3-fold dose escalation above the maximum-tolerated dose [144,145],

although it was concluded that the substantial toxicity, inconvenience and costs associated with this high dose topotecan/G-CSF regimen does not warrant further development. Similarly disappointing results have been obtained with topotecan or 9-AC administered by prolonged continuous infusions [146,147]. The addition of G-CSF with CPT-11 administration has also been advocated by some investigators [148,149], evidence for increased dose intensity or clearly improved chemotherapy based on G-CSF support is still lacking. Therefore, the use of G-CSF outside clinical trials to support chemotherapeutic treatment is not recommended.

The principal non-hematologic toxicity for all topoisomerase I inhibitors is gastro-intestinal. Nausea and vomiting are frequent [56,75,104,112] but, with the introduction of selective serotonine antagonist of the $5HT_3$ –receptor, this side effect is adequately manageable. Diarrhea is also frequent, and mild to moderate in severity with i.v. administration schedules. It appears to be unrelated to the schedule used [56,75,104,112], except in case of CPT-11, where with the oral administration diarrhea becomes the prime dose limiting toxicity especially when using the prolonged schedules [24,122,125,126,150].

In the treatment with CPT-11 two types of diarrhea can be distinguished, *viz.* an early and a late onset form. The early onset diarrhea is part of a cholinergic-like syndrome and manifests in sweating, salivation, abdominal cramping and diarrhea [151]. Interestingly, this cholinergic syndrome has not been described for other CPT analogues. It has been argued that the unique structural features of CPT-11, including a bipiperidino group that shows similarity with a known stimulant of nicotine receptors of autonomic ganglia, dimethylphenylpiperazinium iodide, is responsible for this phenomenon [152]. More recently, it has been found that the mechanism behind the transient cholinerigic reaction observed clinically is more likely mediated through a rapid reversible inhibition of acetylcholinesterase by the lactone form of CPT-11 [153]. Clinical evidence indicates that this side effect can be adequately treated in the acute phase as well as prophylactically with the use of i.v. atropine.

CPT-11-induced delayed type diarrhea has been reported to be severe (NCI-CTC grade 3-4) in 11-23% of the patients [113,154], but even the less severe diarrhea still might influence continuation of therapy. Moreover, diarrhea related to drug-induced colon-mucosal damage, as observed in both rodents [155] and humans [156], can cause severe and potentially lethal illness especially during concomitant occurrence of neutropenia. Once delayed type diarrhea has occurred, a high dose regimen loperamide renders the diarrhea manageable [157].

Prophylactic treatment of this frequently observed side effect has been investigated in numerous studies. Potential modulation of delayed-type diarrhea has been examined clinically with several agents, including an enkephalinase inhibitor [158] and glutamine [159], and in animal models with a lipopeptide [160] and interleukin 15 [161], with different results. A recent preliminary report on co-treatment with thalidomide claims a good protection against diarrhea, but we still have to await pharmacokinetic results to weigh this study on its proper value [162]. To explain the mechanism by which CPT-11-mediated delayed-type diarrhea is

triggered, many pharmacokinetic analyses in humans have been performed in order to predict the incidence of this diarrhea, with conflicting results. Some studies described a correlation between late-onset diarrhea and the systemic glucuronidation rates of SN-38 (i.e., the biliary index) [163]. Recently it was suggested from animal models, that β-glucuronidase activity from the microflora in the large intestines may play a major role in the development of CPT-11-induced diarrhea, by mediating the hydrolysis of biliary secreted SN-38G, thereby causing local accumulation of SN-38, which subsequently causes damage to the intestinal epithelium [164]. This observation has led to experiments in which antibiotic treatment inhibited the β-glucuronidase activity from the intestinal microflora, thereby decreasing the luminal SN-38 concentration and subsequently reducing intestinal damage and ameliorating diarrhea [155]. A recent study in humans showed that antibiotic treatment with neomycin did not alter SN-38 pharmacokinetics in plasma, and gave protection to recurrent diarrhea in over 85% of the patients experiencing diarrhea in the first course (unpublished data, DK and AS). The expected mechanism of blocking bacterial β-glucuronidase activity causing a subsequent rise in SN-38/SN-38G concentration ratio has recently been confirmed. It would be even more attractive to use an agent that specifically inhibits the microbial β-glucuronidase activity. Hange-shasin-to (also referred to as TJ14), a herbal medicine that contains the β-glucuronidase inhibitor baicalin, has recently been described to be a potent inhibitor of delayed-type diarrhea caused by CPT-11 in a rat model [165] as well as in humans [166]. Unfortunately there is no information yet on possible changes in the systemic disposition of CPT-11 and its metabolites which are likely to occur due to inhibition of plasma β-glucuronidase activity by this agent, information of vital importance in view of antitumor activity.

It has also been speculated based on *in vitro* studies, that raising pH-values in the intestines by intestinal alkalization might decrease reabsorption of biliary secreted SN-38 after i.v. CPT-11 administration and, as a result, lowers intestinal side effects [167]. Again, demonstration of unaltered pharmacokinetics of SN-38 in the presence of intestinal alkalization is of crucial importance. Thus, although this approach might show reduced CPT-11-mediated intestinal toxicity, this may be a phyrric victory if a simultaneously altered metabolic clearance (by way of a decreased enterohepatic recirculation of SN-38) results in reduced antitumor activity.

Pharmacokinetic Alterations

Intestinal metabolic systems and drug efflux pumps, located in the intestinal mucosa represent a major limitation in the bioavailability of orally delivered drugs [168]. Several enzymes located in the enterocyte, like the cytochrome P450-3A4 isozyme (CYP3A4), are involved in the presystemic metabolism of many anticancer agents, including etoposide and paclitaxel, thereby limiting the oral absorption of these drugs. Since CYP3A4 is involved in

the metabolism of CPT-11, it has been proposed that the bioavailability of this agent might be substantially enhanced by pharmacokinetic modulation of enteric CYP3A4 activity, e.g. by concomitant administration of ketoconazole, erythromycin or quinidine [169]. Similarly, P-glycoprotein and the Breast Cancer Resistance Protein (BCRP), which are abundantly present in the gastrointestinal tract, have been shown recently to limit the intestinal absorption of various agents, including topotecan [170]. Combined inhibition of intestinal P-glycoprotein and BCRP by the investigational agent GF120918 was shown recently to increase the systemic exposure to topotecan in both animals and patients with the bioavailability increasing from a mere 30-44% to >90%, suggesting that modulation of these transporters simultaneously could be considered in the development of substrate anticancer agents given by the oral route [171].

As indicated, several preclinical studies have identified CYP3A4, to form two pharmacologically inactive oxidation products known as APC and NPC, as one of the principal enzymes involved in CPT-11. In addition, it was shown that ketoconazole, a synthetic imidazole-type broad-spectrum antifungal agent as well as a potent inhibitor of CYP3A4, inhibited APC and NPC formation by 98 and 99%, respectively, at tested concentrations as low as 1 μM [172,183]. Previous investigations indicated that with standard oral doses of ketoconazole (200 to 400 mg/day), peak plasma concentrations are in the range of 4 to 20 μM, suggesting that concomitant treatment of ketoconazole is likely to substantially alter the disposition of CPT-11 administered i.v. to cancer patients. Indeed, a recent pilot pharmacokinetic study in a cancer patient receiving CPT-11 with or without ketoconazole indicated a substantial pharmacokinetic interaction between the two drugs at the level of drug metabolism, and indicated that these agents can not be administered together without dose adjustments (unpublished data, AS and JV). If confirmed in a larger group of patients, the concomitant administration of ketoconazole might enable CPT-11 dose reductions without affecting systemic exposure to the active metabolite, SN-38, and potentially eliminates interpatient variability in CPT-11 pharmacokinetics that arise as a result of (genetically-defined) patient differences in CYP3A4 expression levels [174].

There are many other potential approaches to improve the therapeutic index of CPT-11 through pharmacokinetic biomodulation, including modulation with inhibitors of biliary secretion processes mediated by P-glycoprotein and/or cMOAT (e.g., cyclosporin A) and with inducers of UDP glucuronosyltransferase isoforms involved in SN-38 glucuronidation (e.g., phenobarbital) [175,176]. A clinical trial to evaluate the pharmacological and toxicological implications of a combination regimen of CPT-11, cyclosporin A and phenobarbital is currently in progress, and preliminary findings indicate substantial antitumor activity with this combination without the occurrence of significant diarrhea despite a very low systemic exposure to SN-38 [177]. This finding is supportive of the conjecture that activation of CPT-11 by intratumoral carboxylesterases might be even more important that circulating SN-38 concentrations [178,179].

Conclusions and Future Perspectives

Camptothecins are among the most promising new anticancer drugs that have been developed in recent years. Topotecan and CPT-11 are now registered for the treatment of ovarian and colon cancer, respectively. The unique mechanism of action on topoisomerase I and activity against a broad spectrum of other malignancies are an ongoing stimulus for further clinical development. With the growing knowledge on pharmacodynamics and pharmacokinetics of the different campotothecin analogues, the poor water solubility and pH-dependent reversible interconvention between the active lactone and inactive carboxylate form, as well as increase in activity or stability by substitutions to specific sites on the molecule, much effort has and will be done to increase antitumor activity of this group of drugs. Meanwhile pharmacological modulation, particularly of CPT-11, can be of interest to reduce toxicity and to influence metabolic pathways. Although much effort is being put into development of new analogues, the question to answer remains if drugs under development will ultimately lead to the theoretically expected higher activity and/or reduced toxicity. Last but not least optimization of schedules, routes of administration and combination therapies will lead to numerous new studies in different tumor types. It is to be expected that in the future many new formulations and/or combinations will be developed. In contrast to other previously registered anticancer drugs, the pharmacological knowledge on how camptothecin analogues behave in humans will lead to a more logic and quicker development of these agents.

References

1. Wall ME. Camptothecin and taxol: discovery to clinic. Med Res Rev. 1998;18:299-314.
2. Muggia FM, Creaven PJ, Hansen HH, et al. Phase I clinical trial of weekly and daily treatment with camptothecin (NSC-100880): correlation with preclinical studies. Cancer Chemother Rep. 1972;56:515-521.
3. Schaeppi U, Fleischman RW, Cooney DA. Toxicity of camptothecin (NSC-100880). Cancer Chemother Rep. 1974;5:25-36.
4. Moertel CG, Schutt AJ, Reitemeier RJ, et al. Phase II study of camptothecin (NSC-100880) in the treatment of advanced gastrointestinal cancer. Cancer Chemother Rep. 1972;56:95-101.
5. Creemers GJ, Lund B, Verweij J. Topoisomerase I inhibitors: topotecan and irenotecan. Cancer Treat Rev. 1994;20:73-96.
6. Hsiang YH, Lihou MG, Liu LF. Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. Cancer Res. 1989;49:5077-5082.
7. Hsiang YH, Hertzberg R, Hecht S, et al. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J Biol Chem. 1985; 260:14873-14878.
8. Hsiang YH, Liu LF. Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. Cancer Res. 1988;48:1722-1726.
9. Giovanella BC, Stehlin JS, Wall ME, et al. DNA topoisomerase I--targeted chemotherapy of human colon cancer in xenografts . Science. 1989;246:1046-1048.

10. Van der Zee AG, Hollema H, de Jong S, et al. P-glycoprotein expression and DNA topoisomerase I and II activity in benign tumors of the ovary and in malignant tumors of the ovary, before and after platinum/cyclophosphamide chemotherapy. Cancer Res. 1991;51:5915-5920.
11. Potmesil M. Camptothecins: from bench research to hospital wards. Cancer Res. 1994;54:1431-1439.
12. Van Cutsem E, Cunningham D, Bokkel Huinink WW, et al. Clinical activity and benefit of irinotecan (CPT-11) in patients with colorectal cancer truly resistant to 5-fluorouracil (5-FU). Eur J Cancer. 1999;35:54-59.
13. ten Bokkel HW, Gore M, Carmichael J, et al. Topotecan versus paclitaxel for the treatment of recurrent epithelial ovarian cancer [see comments]. J Clin Oncol. 1997;15:2183-2193.
14. Shimada Y, Rothenberg M, Hilsenbeck SG, et al. Activity of CPT-11 (irinotecan hydrochloride), a topoisomerase I inhibitor, against human tumor colony-forming units. Anticancer Drugs. 1994;5:202-206.
15. Hare CB, Elion GB, Houghton PJ, et al. Therapeutic efficacy of the topoisomerase I inhibitor 7-ethyl-10-(4-[1- piperidino]-1-piperidino)-carbonyloxy-camptothecin against pediatric and adult central nervous system tumor xenografts. Cancer Chemother Pharmacol. 1997;39:187-191.
16. Tanizawa A, Fujimori A, Fujimori Y, et al. Comparison of topoisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin derivatives presently in clinical trials. J Natl Cancer Inst. 1994;86:836-842.
17. Humerickhouse R, Lohrbach K, Li L, et al. Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. Cancer Res. 2000;60:1189-1192.
18. Iyer L, King CD, Whitington PF, et al. Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. J Clin Invest. 1998;101:847-854.
19. Burris HA, III, Hanauske AR, Johnson RK, et al. Activity of topotecan, a new topoisomerase I inhibitor, against human tumor colony-forming units in vitro. J Natl Cancer Inst. 1992;84:1816-1820.
20. Houghton PJ, Cheshire PJ, Hallman JD, et al. Efficacy of topoisomerase I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. Cancer Chemother Pharmacol. 1995; 36:393-403.
21. Daoud SS, Fetouh MI, Giovanella BC. Antitumor effect of liposome-incorporated camptothecin in human malignant xenografts . Anticancer Drugs. 1995;6:83-93.
22. Giovanella BC, Hinz HR, Kozielski AJ, et al. Complete growth inhibition of human cancer xenografts in nude mice by treatment with 20-(S)-camptothecin. Cancer Res. 1991;51:3052-3055.
23. Pantazis P, Hinz HR, Mendoza JT, et al. Complete inhibition of growth followed by death of human malignant melanoma cells in vitro and regression of human melanoma xenografts in immunodeficient mice induced by camptothecins. Cancer Res. 1992;52:3980-3987.
24. de Jonge MJ, Punt CJ, Gelderblom AH, et al. Phase I and pharmacologic study of oral (PEG-1000) 9-aminocamptothecin in adult patients with solid tumors. J Clin Oncol. 1999;17:2219-2226.
25. Hochster H, Liebes L, Speyer J, et al. Phase I trial of low-dose continuous topotecan infusion in patients with cancer: an active and well-tolerated regimen. J Clin Oncol. 1994;12:553-559.
26. Herben VM, Schellens JH, Swart M, et al. Phase I and pharmacokinetic study of irinotecan administered as a low- dose, continuous intravenous infusion over 14 days in patients with malignant solid tumors. J Clin Oncol. 1999;17:1897-1905.

27. Herben VM, Bokkel Huinink WW, Schot ME, et al. Continuous infusion of low-dose topotecan: pharmacokinetics and pharmacodynamics during a phase II study in patients with small cell lung cancer. Anticancer Drugs. 1998;9:411-418.
28. Kindler HL, Kris MG, Smith IE, et al. Phase II trial of topotecan administered as a 21-day continuous infusion in previously untreated patients with stage IIIB and IV non- small-cell lung cancer. Am J Clin Oncol. 1998;21:438-441.
29. Creemers GJ, Gerrits CJ, Schellens JH, et al. Phase II and pharmacologic study of topotecan administered as a 21-day continuous infusion to patients with colorectal cancer. J Clin Oncol. 1996;14:2540-2545.
30. Chourpa I, Beljebbar A, Sockalingum GD, et al. Structure-activity relation in camptothecin antitumor drugs: why a detailed molecular characterisation of their lactone and carboxylate forms by Raman and SERS spectroscopies? Biochim Biophys Acta. 1997;1334:349-360.
31. Takimoto CH, Wright J, Arbuck SG. Clinical applications of the camptothecins. Biochim Biophys Acta. 1998;1400:107-119.
32. Sugimori M, Ejima A, Ohsuki S, et al. Synthesis and antitumor activity of ri. J Med Chem. 1998;41:2308-2318.
33. Luzzio MJ, Besterman JM, Emerson DL, et al. Synthesis and antitumor activity of novel water soluble derivatives of camptothecin as specific inhibitors of topoisomerase I. J Med Chem. 1995;38:395-401.
34. Uehling DE, Nanthakumar SS, Croom D, et al. Synthesis, topoisomerase I inhibitory activity, and in vivo evaluation of 11-azacamptothecin analogs. J Med Chem. 1995;38:1106-1118.
35. Vladu B, Woynarowski JM, Manikumar G, et al. 7- and 10-substituted camptothecins: dependence of topoisomerase I-DNA cleavable complex formation and stability on t. Mol Pharmacol. 2000;57:243-251.
36. Chourpa I, Riou JF, Millot JM, et al. Modulation in kinetics of lactone ring hydrolysis of camptothecins upon interaction with topoisomerase I cleavage sites on DNA. Biochemistry. 1998;37:7284-7291.
37. Burke TG, Munshi CB, Mi Z, et al. The important role of albumin in determining the relative human blood stabilities of the camptothecin anticancer drugs [letter] [published erratum appears in J Pharm Sci 1995 Dec;84(12):1492]. J Pharm Sci. 1995;84:518-519.
38. Loos WJ, Verweij J, Gelderblom HJ, et al. Role of erythrocytes and serum proteins in the kinetic profile of total 9-amino-20(S)-camptothecin in humans. Anticancer Drugs. 1999;10:705-710.
39. Machida Y, Onishi H, Kurita A, et al. Pharmacokinetics of prolonged-release CPT-11-loaded microspheres in rats. J Control Release. 2000;66:159-175.
40. Shenderova A, Burke TG, Schwendeman SP. The acidic microclimate in poly(lactide-co-glycolide) microspheres stabilizes camptothecins. Pharm Res. 1999;16:241-248.
41. Shenderova A, Burke TG, Schwendeman SP. Stabilization of 10-hydroxycamptothecin in poly(lactide-co-glycolide) microsphere delivery vehicles. Pharm Res. 1997;14:1406-1414.
42. Ertl B, Platzer P, Wirth M, et al. Poly(D,L-lactic-co-glycolic acid) microspheres for sustained delivery and stabilization of camptothecin. J Controlled Release. 1999;61:305-317.
43. Shenderova A, Burke TG, Schwendeman SP. The acidic microclimate in poly(lactide-co-glycolide) microspheres stabilizes camptothecins. Pharm Res. 1999;16:241-248.
44. Subramanian D, Muller MT. Liposomal encapsulation increases the activity of the topoisomerase I inhibitor topotecan. Oncol Res. 1995;7:461-469.
45. Burke TG, Gao X. Stabilization of topotecan in low pH liposomes composed of distearoylphosphatidylcholine. J Pharm Sci . 1994;83:967-969.
46. Sadzuka Y, Hirotsu S, Hirota S. Effect of liposomalization on the antitumor activity, side-effects and tissue distribution of CPT-11. Cancer Lett. 1998;127:99-106.

47. Sadzuka Y, Hirotsu S, Hirota S. Effective irinotecan (CPT-11)-containing liposomes: intraliposomal conversion to the active metabolite SN-38. Jpn J Cancer Res. 1999;90:226-232.
48. Tardi P, Choice E, Masin D, et al. Liposomal encapsulation of topotecan enhances anticancer efficacy in murine and human xenograft models. Cancer Res. 2000;60:3389-3393.
49. Emerson DL, Bendele R, Brown E, et al. Antitumor efficacy, pharmacokinetics, and biodistribution of NX 211: a low-clearance liposomal formulation of lurtotecan. Clin Cancer Res. 2000;6:2903-2912.
50. Colbern GT, Dykes DJ, Engbers C, et al. Encapsulation of the topoisomerase I inhibitor GL147211C in pegylated (STEALTH) liposomes: pharmacokinetics and antitumor activity in HT29 colon tumor xenografts. Clin Cancer Res. 1998;4:3077-3082.
51. Loos WJ, Kehrer D, Brouwer E, et al. Liposomal lurtotecan (NX211): determination of total drug levels in human plasma and urine by reversed-phase high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 2000;738:155-163.
52. Yang SC, Lu LF, Cai Y, et al. Body distribution in mice of intravenously injected camptothecin solid lipid nanoparticles and targeting effect on brain. J Controlled Release. 1999;59:299-307.
53. Underberg WJ, Goossen RM, Smith BR, et al. Equilibrium kinetics of the new experimental anti-tumour compound SK&F 104864-A in aqueous solution. J Pharm Biomed Anal. 1990;8:681-683.
54. Saltz LB, Kemeny NE, Tong W, et al. 9-Aminocamptothecin by 72-hour continuous intravenous infusion is inactive in the treatment of patients with 5-fluorouracil-refractory colorectal carcinoma. Cancer. 1997;80:1727-1732.
55. Rubin E, Wood V, Bharti A, et al. A phase I and pharmacokinetic study of a new camptothecin derivative, 9- aminocamptothecin. Clin Cancer Res. 1995;1:269-276.
56. Dahut W, Harold N, Takimoto C, et al. Phase I and pharmacologic study of 9-aminocamptothecin given by 72-hour infusion in adult cancer patients. J Clin Oncol. 1996; 14:1236-1244.
57. Takimoto CH, Dahut W, Marino MT, et al. Pharmacodynamics and pharmacokinetics of a 72-hour infusion of 9- aminocamptothecin in adult cancer patients. J Clin Oncol. 1997;15:1492-1501.
58. Siu LL, Oza AM, Eisenhauer EA, et al. Phase I and pharmacologic study of 9-aminocamptothecin colloidal dispersion formulation given as a 24-hour continuous infusion weekly times four every 5 weeks. J Clin Oncol. 1998;16:1122-1130.
59. Kraut EH, Balcerzak SP, Young D, et al. A phase II study of 9-aminocamptothecin in patients with refractory breast cancer. Cancer Invest. 2000;18:28-31.
60. Vey N, Kantarjian H, Tran H, et al. Phase I and pharmacologic study of 9-aminocamptothecin colloidal dispersion formulation in patients with refractory or relapsed acute leukemia. Ann Oncol. 1999;10:577-583.
61. Herben VM, van Gijn R, Schellens JH, et al. Phase I and pharmacokinetic study of a daily times 5 short intravenous infusion schedule of 9-aminocamptothecin in a colloidal dispersion formulation in patients with advanced solid tumors. J Clin Oncol. 1999;17:1906-1914.
62. Pazdur R, Diaz-Canton E, Ballard WP, et al. Phase II trial of 9-aminocamptothecin administered as a 72-hour continuous infusion in metastatic colorectal carcinoma. J Clin Oncol . 1997;15:2905-2909.
63. Pazdur R, Medgyesy DC, Winn RJ, et al. Phase II trial of 9-aminocamptothecin (NSC 603071) administered as a 120-hr continuous infusion weekly for three weeks in metastatic colorectal carcinoma. Invest New Drugs. 1998;16:341-346.
64. Vokes EE, Ansari RH, Masters GA, et al. A phase II study of 9-aminocamptothecin in advanced non-small-cell lung cancer [see comments]. Ann Oncol. 1998;9 :1085-1090.
65. Lavergne O, Lesueur-Ginot L, Pla RF, et al. Homocamptothecins: synthesis and antitumor activity of novel E-ring- modified camptothecin analogues. J Med Chem. 1998;41:5410-5419.

66. Lavergne O, Demarquay D, Bailly C, et al. Topoisomerase I-mediated antiproliferative activity of enantiomerically pure fluorinated homocamptothecins. J Med Chem. 2000;43:2285-2289.
67. Lesueur-Ginot L, Demarquay D, Kiss R, et al. Homocamptothecin, an E-ring modified camptothecin with enhanced lactone stability, retains topoisomerase I-targeted activity and antitumor properties. Cancer Res. 1999;59:2939-2943.
68. Philippart P, Harper L, Chaboteaux C, et al. Homocamptothecin, an E-ring-modified camptothecin, exerts more potent antiproliferative activity than other topoisomerase I inhibitors in human colon cancers obtained from surgery and maintained in vitro under histotypical culture conditions. Clin Cancer Res. 2000;6:1557-1562.
69. Bailly C, Lansiaux A, Dassonneville L, et al. Homocamptothecin, an E-ring-modified camptothecin analogue, generates new topoisomerase I-mediated DNA breaks. Biochemistry. 1999;38:15556-15563.
70. Lesueur-Ginot L, Demarquay D, Kiss R, et al. Homocamptothecin, an E-ring modified camptothecin with enhanced lactone stability, retains topoisomerase I-targeted activity and antitumor properties. Cancer Res. 1999;59:2939-2943.
71. Lavergne O, Bigg DC. [The other camptothecins: recent advances with camptothecin analogues other than irinotecan and topotecan]. Bull Cancer. 1998;Spec No:51-58.
72. Lavergne O, Harnett J, Rolland A, et al. BN 80927: a novel homocamptothecin with inhibitory activities on both topoisomerase I and topoisomerase II. Bioorg Med Chem Lett. 1999;9:2599-2602.
73. Pollack IF, Erff M, Bom D, et al. Potent topoisomerase I inhibition by novel silatecans eliminates glioma proliferation in vitro and in vivo. Cancer Res. 1999;59:4898-4905.
74. Emerson DL, Besterman JM, Brown HR, et al. In vivo antitumor activity of two new seven-substituted water-soluble camptothecin analogues. Cancer Res. 1995;55:603-609.
75. Gerrits CJ, Creemers GJ, Schellens JH, et al. Phase I and pharmacological study of the new topoisomerase I inhibitor GI147211, using a daily x 5 intravenous administration. Br J Cancer. 1996;73:744-750.
76. Eckhardt SG, Baker SD, Eckardt JR, et al. Phase I and pharmacokinetic study of GI147211, a water-soluble camptothecin analogue, administered for five consecutive days every three weeks. Clin Cancer Res. 1998;4:595-604.
77. Paz-Ares L, Kunka R, DeMaria D, et al. A phase I clinical and pharmacokinetic study of the new topoisomerase inhibitor GI147211 given as a 72-h continuous infusion. Br J Cancer . 1998;78:1329-1336.
78. Gerrits CJ, Schellens JH, Creemers GJ, et al. The bioavailability of oral GI147211 (GG211), a new topoisomerase I inhibitor. Br J Cancer. 1997;76:946-951.
79. Mitsui I, Kumazawa E, Hirota Y, et al. A new water-soluble camptothecin derivative, DX-8951f, exhibits potent antitumor activity against human tumors in vitro and in vivo. Jpn J Cancer Res. 1995;86:776-782.
80. Lawrence RA, Izbicka E, De Jager RL, et al. Comparison of DX-8951f and topotecan effects on tumor colony formation from freshly explanted adult and pediatric human tumor cells. Anticancer Drugs. 1999;10:655-661.
81. Vey N, Giles FJ, Kantarjian H, et al. The topoisomerase I inhibitor DX-8951f is active in a severe combined immunodeficient mouse model of human acute myelogenous leukemia. Clin Cancer Res. 2000;6:731-736.
82. Rowinsky EK, Johnson TR, Geyer CE, Jr., et al. DX-8951f, a hexacyclic camptothecin analog, on a daily-times-five schedule: a phase I and pharmacokinetic study in patients with advanced solid malignancies. J Clin Oncol. 2000;18:3151-3163.
83. Kamiya Y, Yamamoto N, Yamada Y, et al. Phase I and pharmacokinetic (PK) study of DX-8951f, anovel camptothecin analog, given as a 30 minute infusion daily for 5 days. Clin.Cancer Res. 2000;5(S):327.

84. Boige V, Raymond E, Gatineau M, et al. Final results of a phase I and pharmacokinetic study of DX-8951f in patients with advanced tumors. Proc.Am.Assoc.Cancer Res. 2000;40:754.
85. Minami H, Sasaki Y, Shigeoka Y, et al. Phase I study and pharmacology of DX-8951f, a new camptothecin derivative administered over 30 minutes every 3 weeks. Clin.Cancer Res. 2000;5(S):326.
86. Garrison M, Hammond L, Geyer C, et al. A phase I and pharmacokinetic study of the camptothecin (CPT) analog DX-8951f (exetacan mesylate): escalating infusion duration and dose. Proc.Am.Soc.Clin.Oncol. 2000;19:765.
87. Talbot DC, White S, Jones P, et al. Phase II study of exatecan mesylate (DX-8951f) in advanced NSCLC. Proc.Am.Soc.Clin.Oncol. 2000;19:2166.
88. O'Reilly EM, Hammond L, Sharma S, et al. A phase II study of exatecan mesylate (DX-8951f), DX) in advanced pancreatic cancer. Proc.Am.Soc.Clin.Oncol. 2000;19:1170.
89. Kudelka AP, Verschraegen C, Vincent MD, et al. Phase II study of intravenous DX-8951f in patients (Pts) with advanced ovarian, tubal, or peritoneal cancer refractory to platinum, taxane, and topotecan. Proc.Am.Soc.Clin.Oncol. 2000;19:1550.
90. Royce M, Saltz L, Rowinsky EK, et al. A phase II study of intravenous exatecan mesylate (Dx-8951f, Dx) administered daily for five days every three weeks to patients with advanced or metastatic adenocarcinoma of the colon or rectum. Proc.Am.Soc.Clin.Oncol. 2000;19:1129.
91. Karaberis E, Mourelatos D. Enhanced cytogenetic and antitumor effects by 9-nitrocamptothecin and antineoplastics. Teratog Carcinog Mutagen. 2000;20:141-146.
92. Pantazis P, Early JA, Mendoza JT, et al. Cytotoxic efficacy of 9-nitrocamptothecin in the treatment of human malignant melanoma cells in vitro. Cancer Res. 1994;54 :771-776.
93. Hinz HR, Harris NJ, Natelson EA, et al. Pharmacokinetics of the in vivo and in vitro conversion of 9-nitro- 20(S)-camptothecin to 9-amino-20(S)-camptothecin in humans, dogs, and mice. Cancer Res. 1994;54:3096-3100.
94. Gelderblom HA, de Jonge MJ, Sparreboom A, et al. Oral topoisomerase 1 inhibitors in adult patients: present and future. Invest New Drugs. 1999;17:401-415.
95. Stehlin JS, Giovanella BC, Natelson EA, et al. A study of 9-nitrocamptothecin (RFS-2000) in patients with advanced pancreatic cancer. Int J Oncol. 1999;14:821-831.
96. Verschraegen CF, Gupta E, Loyer E, et al. A phase II clinical and pharmacological study of oral 9- nitrocamptothecin in patients with refractory epithelial ovarian, tubal or peritoneal cancer. Anticancer Drugs. 1999;10:375-383.
97. Conover CD, Greenwald RB, Pendri A, et al. Camptothecin delivery systems: the utility of amino acid spacers for the conjugation of camptothecin with polyethylene glycol to create prodrugs. Anticancer Drug Des. 1999;14:499-506.
98. Conover CD, Pendri A, Lee C, et al. Camptothecin delivery systems: the antitumor activity of a camptothecin- 20-0-polyethylene glycol ester transport form. Anticancer Res. 1997;17:3361-3368.
99. Maeda H, Matsumura Y. Tumoritropic and lymphotropic principles of macromolecular drugs. Crit Rev Ther Drug Carrier Syst. 1989;6:193-210.
100. Okuno S, Harada M, Yano T, et al. Complete regression of xenografted human carcinomas by camptothecin analogue-carboxymethyl dextran conjugate (T-0128). Cancer Res. 2000;60:2988-2995.
101. Caiolfa VR, Zamai M, Fiorino A, et al. Polymer-bound camptothecin: initial biodistribution and antitumour activity studies. J Controlled Release. 2000;65:105-119.
102. Ochoa L, Tolcher A, Rizzo J, et al. A phase I study of PEG-camptothecin (PEG-CPT) in patients with advanced solid tumors: a novel formulation for an insoluble but active agent. Proc.Am.Soc.Clin.Oncol. 2000;19:198a.

103. de Bono JS, Bisset D, Twelves C, et al. Phase I pharmacokinetic (PK) study of MAG-CPT (PNU 166148), a polymeric derivative of camptothecin (CPT). Proc.Am.Soc.Clin.Oncol. 2000;19:198a.
104. Verweij J, Lund B, Beijnen J, et al. Phase I and pharmacokinetics study of topotecan, a new topoisomerase I inhibitor. Ann Oncol. 1993;4:673-678.
105. Rowinsky EK, Kaufmann SH, Baker SD, et al. A phase I and pharmacological study of topotecan infused over 30 minutes for five days in patients with refractory acute leukemia. Clin Cancer Res. 1996;2:1921-1930.
106. Haas NB, LaCreta FP, Walczak J, et al. Phase I/pharmacokinetic study of topotecan by 24-hour continuous infusion weekly . Cancer Res. 1994;54:1220-1226.
107. van Warmerdam LJ, Bokkel Huinink WW, Rodenhuis S, et al. Phase I clinical and pharmacokinetic study of topotecan administered by a 24-hour continuous infusion. J Clin Oncol. 1995;13:1768-1776.
108. Hochster H, Wadler S, Runowicz C, et al. Activity and pharmacodynamics of 21-Day topotecan infusion in patients with ovarian cancer previously treated with platinum-based chemotherapy. New York Gynecologic Oncology Group. J Clin Oncol. 1999;17:2553-2561.
109. Hoskins P, Eisenhauer E, Beare S, et al. Randomized phase II study of two schedules of topotecan in previously treated patients with ovarian cancer: a National Cancer Institute of Canada Clinical Trials Group study. J Clin Oncol. 1998;16:2233-2237.
110. Mainwaring PN, Nicolson MC, Hickish T, et al. Continuous infusional topotecan in advanced breast and non-small-cell lung cancer: no evidence of increased efficacy. Br J Cancer . 1997;76:1636-1639.
111. Kollmannsberger C, Mross K, Jakob A, et al. Oncology. 1999;56:1-12.
112. Abigerges D, Chabot GG, Armand JP, et al. Phase I and pharmacologic studies of the camptothecin analog irinotecan administered every 3 weeks in cancer patients. J Clin Oncol. 1995;13:210-221.
113. Rothenberg ML, Eckardt JR, Kuhn JG, et al. Phase II trial of irinotecan in patients with progressive or rapidly recurrent colorectal cancer. J Clin Oncol. 1996;14:1128-1135.
114. de Forni M, Bugat R, Chabot GG, et al. Phase I and pharmacokinetic study of the camptothecin derivative irinotecan, administered on a weekly schedule in cancer patients. Cancer Res. 1994;54:4347-4354.
115. Armand JP. CPT-11: clinical experience in phase I studies. Semin Oncol. 1996;23:27-33.
116. Takimoto CH, Morrison G, Harold N, et al. Phase I and pharmacologic study of irinotecan administered as a 96-hour infusion weekly to adult cancer patients. J Clin Oncol. 2000;18:659-667.
117. Ohe Y, Sasaki Y, Shinkai T, et al. Phase I study and pharmacokinetics of CPT-11 with 5-day continuous infusion. J Natl Cancer Inst. 1992;84:972-974.
118. Schellens JH, Creemers GJ, Beijnen JH, et al. Bioavailability and pharmacokinetics of oral topotecan: a new topoisomerase I inhibitor. Br J Cancer. 1996;73:1268-1271.
119. Herben VM, Rosing H, Bokkel Huinink WW, et al. Oral topotecan: bioavailablity and effect of food co-administration. Br J Cancer. 1999;80:1380-1386.
120. Zamboni WC, Bowman LC, Tan M, et al. Interpatient variability in bioavailability of the intravenous formulation of topotecan given orally to children with recurrent solid tumors. Cancer Chemother Pharmacol. 1999;43:454-460.
121. Sparreboom A, de Jonge MJ, Punt CJ, et al. Pharmacokinetics and bioavailability of oral 9-aminocamptothecin capsules in adult patients with solid tumors. Clin Cancer Res. 1998;4:1915-1919.
122. Mani S, Iyer L, Janisch L, et al. Phase I clinical and pharmacokinetic study of oral 9-aminocamptothecin (NSC-603071) . Cancer Chemother Pharmacol. 1998;42:84-87.

123. Stewart CF, Zamboni WC, Crom WR, et al. Disposition of irinotecan and SN-38 following oral and intravenous irinotecan dosing in mice. Cancer Chemother Pharmacol. 1997;40:259-265.
124. Gerrits CJ, Burris H, Schellens JH, et al. Five days of oral topotecan (Hycamtin), a phase I and pharmacological study in adult patients with solid tumours. Eur J Cancer. 1998;34:1030-1035.
125. Gerrits CJ, Burris H, Schellens JH, et al. Oral topotecan given once or twice daily for ten days: a phase I pharmacology study in adult patients with solid tumors. Clin Cancer Res. 1998;4:1153-1158.
126. Creemers GJ, Gerrits CJ, Eckardt JR, et al. Phase I and pharmacologic study of oral topotecan administered twice daily for 21 days to adult patients with solid tumors. J Clin Oncol. 1997;15:1087-1093.
127. Gerrits CJ, Schellens JH, Burris H, et al. A comparison of clinical pharmacodynamics of different administration schedules of oral topotecan (Hycamtin) [see comments]. Clin Cancer Res. 1999;5:69-75.
128. von Pawel J, Gatzemeier U, Harstrick A, et al. A multicentre randomised phase II study of oral topotecan versuis IV topotecan for second line therapy i sensitive patients with small cell lung cancer. Proc Am Soc Clin Oncol. 1998;17:1816.
129. Gore M, Rustin G, Calvert AH, et al. A multicentre, randomised, phase III study of topotecan (T) administered intravenously or orally for advanced epithelial ovarian carcinoma. Proc Am Soc Clin Oncol. 1998;17:1346.
130. Guichard S, Terret C, Hennebelle I, et al. CPT-11 converting carboxylesterase and topoisomerase activities in tumour and normal colon and liver tissues. Br J Cancer. 1999;80:364-370.
131. Thompson J, Zamboni WC, Cheshire PJ, et al. Efficacy of oral irinotecan against neuroblastoma xenografts. Anticancer Drugs. 1997;8:313-322.
132. Zamboni WC, Houghton PJ, Thompson J, et al. Altered irinotecan and SN-38 disposition after intravenous and oral administration of irinotecan in mice bearing human neuroblastoma xenografts. Clin Cancer Res. 1998;4:455-462.
133. Drengler RL, Kuhn JG, Schaaf LJ, et al. Phase I and pharmacokinetic trial of oral irinotecan administered daily for 5 days every 3 weeks in patients with solid tumors. J Clin Oncol. 1999;17:685-696.
134. Guthrie TH, Sanal SM, Agaliotis DP, et al Hepatic artery chemoembolization (CE) with irinotecan (I) for primary and metstatic liver cancer; Preliminary results. Proc Am Soc Clin Oncol. 2000;19:305a.
135. Van der Vijgh WJ, Van Groeningen C, Kedde MA, et al. Pharmacokinetic and pharmacodynamics of CPT-11 during 5-day hepatic arterial (HAI) and intravenous (i.v.) infusion. Proc Am Soc Clin Oncol. 2000;39:600.
136. Knight V, Koshkina NV, Waldrep JC, et al. Anticancer effect of 9-nitrocamptothecin liposome aerosol on human cancer xenografts in nude mice. Cancer Chemother Pharmacol. 1999; 44:177-186.
137. Koshkina NV, Kleinerman ES, Waidrep C, et al. 9-Nitrocamptothecin liposome aerosol treatment of melanoma and osteosarcoma lung metastases in mice. Clin Cancer Res. 2000;6:2876-2880.
138. Koshkina NV, Gilbert BE, Waldrep JC, et al. Distribution of camptothecin after delivery as a liposome aerosol or following intramuscular injection in mice. Cancer Chemother Pharmacol. 1999;44:187-192.
139. Knight V, Koshkina N, Waldrep C, et al. Anti-cancer activity of 9-nitrocamptothecin liposome aerosol in mice. Trans Am Clin Climatol Assoc. 2000;111:135-145.
140. Plaxe SC, Christen RD, O'Quigley J, et al. Phase I and pharmacokinetic study of intraperitoneal topotecan. Invest New Drugs. 1998;16:147-153.

141. Masson E, Zamboni WC. Pharmacokinetic optimisation of cancer chemotherapy. Effect on outcomes. Clin Pharmacokinet. 1997;32:324-343.
142. Saltz L, Janik JE. Topotecan and the treatment of recurrent ovarian cancer: is there a role for granulocyte colony-stimulating factor? Semin Oncol. 1997;24:S5.
143. Saltz L, Sirott M, Young C, et al. Phase I clinical and pharmacology study of topotecan given daily for 5 consecutive days to patients with advanced solid tumors, with attempt at dose intensification using recombinant granulocyte colony- stimulating factor [published erratum appears in J Natl Cancer Inst 1993 Nov 3;85(21):1777]. J Natl Cancer Inst. 1993;85:1499-1507.
144. Rowinsky EK, Baker SD, Burks K, et al. High-dose topotecan with granulocyte-colony stimulating factor in fluoropyrimidine-refractory colorectal cancer: a phase II and pharmacodynamic study. Ann Oncol. 1998;9:173-180.
145. Rowinsky EK, Grochow LB, Sartorius SE, et al. Phase I and pharmacologic study of high doses of the topoisomerase I inhibitor topotecan with granulocyte colony-stimulating factor in patients with solid tumors. J Clin Oncol. 1996;14 :1224-1235.
146. Abbruzzese JL, Madden T, Sugarman SM, et al. Phase I clinical and plasma and cellular pharmacological study of topotecan without and with granulocyte colony-stimulating factor. Clin Cancer Res. 1996;2:1489-1497.
147. Wilson WH, Little R, Pearson D, et al. Phase II and dose-escalation with or without granulocyte colony- stimulating factor study of 9-aminocamptothecin in relapsed and refractory lymphomas [published errata appear in J Clin Oncol 1998 Aug;16(8):2895 and 1999 Jun;17(6):1964]. J Clin Oncol. 1998;16:2345-2351.
148. Masuda N, Fukuoka M, Kudoh S, et al. Phase I and pharmacologic study of irinotecan and etoposide with recombinant human granulocyte colony-stimulating factor support for advanced lung cancer. J Clin Oncol. 1994;12:1833-1841.
149. Fujita A, Takabatake H, Tagaki S, et al. Phase I/II study of cisplatin, ifosfamide and irinotecan with rhG-CSF support in patients with stage IIIB and IV non-small-cell lung cancer. Cancer Chemother Pharmacol. 2000;45:279-283.
150. Verschraegen CF, Natelson EA, Giovanella BC, et al. A phase I clinical and pharmacological study of oral 9- nitrocamptothecin, a novel water-insoluble topoisomerase I inhibitor. Anticancer Drugs. 1998;9:36-44.
151. Bleiberg H, Cvitkovic E. Characterisation and clinical management of CPT-11 (irinotecan)-induced adverse events: the European perspective. Eur J Cancer. 1996;32A Suppl 3:S18-S23.
152. Gandia D, Abigerges D, Armand JP, et al. CPT-11-induced cholinergic effects in cancer patients [letter]. J Clin Oncol. 1993;11:196-197.
153. Dodds HM, Rivory LP. The mechanism for the inhibition of acetylcholinesterases by irinotecan (CPT-11). Mol Pharmacol. 1999;56:1346-1353.
154. Rougier P, Bugat R, Douillard JY, et al. Phase II study of irinotecan in the treatment of advanced colorectal cancer in chemotherapy-naive patients and patients pretreated with fluorouracil-based chemotherapy. J Clin Oncol. 1997;15 :251-260.
155. Takasuna K, Hagiwara T, Hirohashi M, et al. Inhibition of intestinal microflora beta-glucuronidase modifies the distribution of the active metabolite of the antitumor agent, irinotecan hydrochloride (CPT-11) in rats. Cancer Chemother Pharmacol. 1998;42:280-286.
156. Van Huyen JP, Bloch F, Attar A, et al. Diffuse mucosal damage in the large intestine associated with Irinotecan (CPT-11). Dig Dis Sci. 1998;43:2649-2651.
157. Abigerges D, Armand JP, Chabot GG, et al. Irinotecan (CPT-11) high-dose escalation using intensive high-dose loperamide to control diarrhea. J Natl Cancer Inst. 1994;86:446-449.
158. Ychou M, Douillard JY, Rougier P, et al. Randomized comparison of prophylactic antidiarrheal treatment versus no prophylactic antidiarrheal treatment in patients receiving CPT-11 (irinotecan) for advanced 5-FU-resistant colorectal cancer: an open- label multicenter phase II study. Am J Clin Oncol. 2000;23:143-148.

159. Savarese D, Al Zoubi A, Boucher J. Glutamine for irinotecan diarrhea [letter]. J Clin Oncol. 2000;18:450-451.
160. Shinohara H, Killion JJ, Kuniyasu H, et al. Prevention of intestinal toxic effects and intensification of irinotecan's therapeutic efficacy against murine colon cancer liver metastases by oral administration of the lipopeptide JBT 3002. Clin Cancer Res. 1998;4:2053-2063.
161. Cao S, Black JD, Troutt AB, et al Interleukin 15 offers selective protection from irinotecan-induced intestinal toxicity in a preclinical animal model. Cancer Res. 1998;58:3270-3274.
162. Govindarajan R, Heaton KM, Broadwater R, et al. Effect of thalidomide on gastrointestinal toxic effects of irinotecan [letter]. Lancet. 2000; 356:566-567.
163. Gupta E, Lestingi TM, Mick R, et al. Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. Cancer Res. 1994;54:3723-3725.
164. Takasuna K, Hagiwara T, Hirohashi M, et al. Involvement of beta-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res. 1996;56:3752-3757.
165. Takasuna K, Kasai Y, Kitano Y, et al. Protective effects of kampo medicines and baicalin against intestinal toxicity of a new anticancer camptothecin derivative, irinotecan hydrochloride (CPT-11), in rats. Jpn J Cancer Res. 1995;86 :978-984.
166. Mori K, Hirose T, Machida S, et al. [Kampo medicines for the prevention of irinotecan-induced diarrhea in advanced non-small cell lung cancer]. Gan To Kagaku Ryoho. 1998;25:1159-1163.
167. Kobayashi K, Bouscarel B, Matsuzaki Y, et al. pH-dependent uptake of irinotecan and its active metabolite, SN-38, by intestinal cells. Int J Cancer. 1999;83:491-496.
168. Benet LZ, Izumi T, Zhang Y, et al. Intestinal MDR transport proteins and P-450 enzymes as barriers to oral drug delivery. J Controlled Release. 1999;62:25-31.
169. Kobayashi K, Jodrell DI, Ratain MJ. Pharmacodynamic-pharmacokinetic relationships and therapeutic drug monitoring. Cancer Surv. 1993;17:51-78.
170. Maliepaard M, van Gastelen MA, de Jong LA, et al. Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. Cancer Res. 1999;59:4559-4563.
171. Scheffer GL, Maliepaard M, Pijnenborg AC, et al. Breast cancer resistance protein is localized at the plasma membrane in mitoxant. Cancer Res. 2000;60:2589-2593.
172. Dodds HM, Haaz MC, Riou JF, et al. Identification of a new metabolite of CPT-11 (irinotecan): pharmacological properties and activation to SN-38. J Pharmacol Exp Ther. 1998;286:578-583.
173. Haaz MC, Rivory L, Riche C, et al. Metabolism of irinotecan (CPT-11) by human hepatic microsomes: participation of cytochrome P-450 3A and drug interactions. Cancer Res. 1998;58:468-472.
174. Santos A, Zanetta S, Cresteil T, et al. Metabolism of irinotecan (CPT-11) by CYP3A4 and CYP3A5 in humans. Clin Cancer Res. 2000;6:2012-2020.
175. Gupta E, Safa AR, Wang X, et al. Pharmacokinetic modulation of irinotecan and metabolites by cyclosporin A. Cancer Res. 1996;56:1309-1314.
176. Gupta E, Wang X, Ramirez J, et al. Modulation of glucuronidation of SN-38, the active metabolite of irinotecan, by valproic acid and phenobarbital. Cancer Chemother Pharmacol. 1997;39:440-444.
177. Iyer L, Ratain MJ. Clinical pharmacology of camptothecins. Cancer Chemother Pharmacol. 1998;42 Suppl:S31-S43.
178. Danks MK, Morton CL, Pawlik CA, et al. Overexpression of a rabbit liver carboxylesterase sensitizes human tumor cells to CPT-11. Cancer Res. 1998;58:20-22.
179. Danks MK, Morton CL, Krull EJ, et al. Comparison of activation of CPT-11 by rabbit and human carboxylesterases for use in enzyme/prodrug therapy. Clin Cancer Res. 1999;5:917-924.

Chapter 2

Liposomal Lurtotecan (NX211): Determination of Total Drug Levels in Human Plasma and Urine by Reversed-phase High-Performance Liquid Chromatography

W.J. Loos, D.F.S. Kehrer, E. Brouwer, J. Verweij, P. de Bruijn, M. Hamilton, S. Gil, K. Nooter, G. Stoter, A. Sparreboom

Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam, the Netherlands. Gilead Sciences Incorporation, Boulder, CO, USA

Journal of Chromatography B, 738: 155-163, 2000

Abstract

Lurtotecan (GI147211; LRT) is a semisynthetic and water-soluble analogue of the topoisomerase I inhibitor camptothecin. To determine whether the therapeutic efficacy of LRT in patients could be improved, the drug was encapsulated in liposomes (NX 211; Gilead Sciences). In order to allow accurate description of the pharmacokinetic behavior of NX 211 in cancer patients, we have developed sensitive RP-HPLC assays with fluorescence detection (λ_{ex}=378 nm; λ_{em}=420 nm) for the determination of total LRT levels in human plasma and urine. Sample pretreatment involved deproteinization with 10% (w/v) aqueous perchloric acid-acetonitrile (2:1, v/v), and chromatographic separations were achieved on an Inertsil-ODS 80A analytical column. The lower limit of quantitation (LLQ) was established at 1.00 ng/ml in plasma (200-µl sample) and at 100 ng/ml in urine (200 µl of 40-fold diluted sample). The within-run and between-run precisions were <7.5%. LRT concentrations in urine <100 ng/ml were determined by a modified procedure comprising a single solvent extraction with *n*-butanol-diethyl ether (3:4, v/v). In this assay, the fluorescence signal of LRT was increased 14-fold prior to detection by post-column exposure to UV light (254 nm) in a photochemical reaction unit. The LLQ of this assay was 0.500 ng/ml (150-µl sample) and the within-run and between-run precisions were <10%.

Introduction

Lurtotecan (7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(*S*)-camptothecin; also known as GI147211; LRT) (Fig. 1) is a novel semisynthetic analogue of camptothecin, a cytotoxic plant alkaloid that was first extracted from the wood and bark of the oriental tree, *Camptotheca acuminata* [1]. The mechanism of action of camptothecin derivatives is based on stabilization of the cleavable complex formed by the intranuclear enzyme topoisomerase I and DNA, and on induction of single-stranded DNA breaks [2]. LRT has previously been shown to have significant activity in both *in vitro* cytotoxicity assays and *in vivo* tumor model systems [3-5], and was recently introduced into clinical trials [6-8].

Clinical pharmacokinetic studies of camptothecin derivatives, including LRT, are complicated by a chemical, pH-dependent instability of the α-hydroxy-δ-lactone moiety in the core structure of the compounds, generating a ring-opened carboxylate form. This lactone functionality undergoes rapid hydrolysis in aqueous solution under physiological conditions, i.e. at pH 7 or above, and results in a virtually complete loss of biological activity [9]. In recent years, considerable effort has been put in the development of alternative formulations that would allow prolonged systemic exposure to the pharmacologically active drug form.

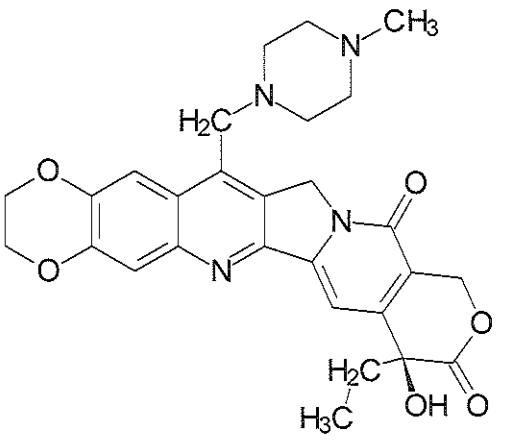


Figure 1. *Chemical structure of lurtotecan (LRT).*

One of these approaches is the incorporation of the lactone forms of camptothecins in liposomal particles. Indeed, recent preclinical studies indicated that liposomal encapsulation of the topoisomerase I inhibitors topotecan [10,11], camptothecin [12,13] and irinotecan [14,15] proved to be very efficient against lactone ring opening, increased antitumor activity in experimental tumor models and dramatically enhanced tissue distribution and the systemic availability. Efficacy studies performed in nude mice bearing human colon and head and neck tumor xenografts have also indicated improved therapeutic efficacy for a new liposomal formulation of LRT (NX 211; Gilead Sciences) as compared to nonliposomal LRT [16].
Based on these favorable results, we recently started a project to study the safety profile and clinical pharmacokinetics of NX 211 in patients with advanced solid tumors. In the context of this study, we have now developed sensitive RP-HPLC methods with fluorescence detection using a sample clean-up procedure that disrupts the liposomes, thus enabling determination of total drug levels in plasma and urine samples following NX 211 administration. The methods have been validated in terms of sensitivity, accuracy and precision [17], and have been used in a pharmacokinetic experiment in a patient to investigate their applicability *in vivo*.

Experimental

Chemicals and Reagents

LRT dihydrochloride monohydrate (lot: U2044/164/1, containing 78.11% of the free base) and NX 211 (liposomal LRT, lot: 181801F, containing 0.49 mg LRT as free base/ml) were delivered by Gilead Sciences (San Dimas, CA, USA). The internal standard (IS) 6,7-

dimethoxy-4-methylcoumarin (lot 79F3652) was obtained from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), diethyl ether, *n*-butanol, methanol and acetonitrile were purchased from Rathburn (Walkerburn, UK). Perchloric acid (70-72%, v/v, in water), neat acetic acid and sodium hydroxide were supplied by Baker (Deventer, The Netherlands). Ammonium acetate was delivered by Roth (Karlsruhe, Germany) and sodium chloride by Merck (Darmstadt, Germany). All water used in the study was filtered and deionized with a Milli-Q-UF system (Millipore, Milford, MA, USA). Drug-free human plasma for the construction of calibration curves and quality control (QC) samples originated from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

Stock Solutions

Stock solutions of LRT were made in triplicate by dissolving X mg LRT in (X x 0.7811) ml DMSO, resulting in a solution containing 1.00 mg/ml LRT (free base). The working stock solution of LRT, containing 0.100 mg/ml free base, was prepared by a 10-fold dilution of the stock solution in DMSO. A stock solution of the IS at a concentration of 1 mg/ml was prepared by dissolving 50 mg IS in 50 ml DMSO.

Standards for Total LRT in Plasma and Urine

Spiked plasma samples used as calibration standards were prepared daily in duplicate by addition of 10 µl of serial dilutions in methanol-water (1:1,v/v) from the working solution of LRT to 240 µl of drug-free human plasma. This resulted in calibration standards of 1.00, 2.50, 5.00, 10.0, 25.0, 50.0 and 100 ng/ml LRT (free base) in plasma. Four pools of QC samples for LRT were prepared in human plasma at concentrations of 4.00, 20.0, 75.0 and 750 ng/ml, by addition of the appropriate volume of the LRT working solution or dilutions in 10 mM aqueous sodium hydroxide-methanol (1:1, v/v) (to shift the equilibrium to the carboxylate form) to human plasma. The QC containing 750 ng/ml LRT was used to investigate the suitability of small-volume (20 µl) injections. Lower limit of quantitation (LLQ) samples in plasma were prepared daily in separate blank plasma samples obtained from 5 healthy volunteers at a concentration of 1.00 ng/ml. To minimize a potential difference with clinical samples, 2 pools of recovery control (RC) samples containing 20.0 and 750 ng/ml NX 211 were also prepared by addition of an aliquot of the NX 211 stock solution (in phosphate-buffered saline) to human plasma. Two pools of QC samples were prepared in human urine at concentrations of 250 and 2500 ng/ml, which were diluted 40-fold in blank human plasma prior to extraction. The LLQ samples for urine were prepared daily at a spiked concentration of 100 ng/ml in quintuplicate using 5 different drug-free urine samples.

Standards of LRT in the Sensitive Urine Assay

Calibration standards in urine were also prepared daily in duplicate by addition of 10 µl of serial dilutions of LRT in methanol-water (1:1, v/v) to 240 µl blank human urine, at final

concentrations of the free base of 0.500, 1.00, 2.50, 5.00 and 10.0 ng/ml. Three pools of QC samples were prepared at concentrations of 1.25, 7.50 and 250 ng/ml by addition of appropriate volumes of a dilution of the LRT working stock solution [in 10 mM aqueous sodium hydroxide-methanol (1:1, v/v)]. The QC sample containing 250 ng/ml, identical to that used for the assay of total LRT in plasma and urine, was diluted 10-fold in blank urine before extraction, and was further used to show the applicability of low volume injections (10 µl). The LLQ samples were prepared daily at a concentration of 0.500 ng/ml, again in quintuplicate using 5 different drug-free urine samples.

HPLC Instrumentation and Conditions

The HPLC systems consisted of constaMetric 3200 and 4100 solvent delivery systems (LDC Analytical, Riviera Beach, FL, USA), Waters 717plus autosampling devices (Milford, MA, USA), a Beam Boost photochemical reaction unit supplied with a coil of 25 m and an internal diameter of 0.3 mm (ICT-ASS-Chem, Bad Homburg, Germany), and Jasco 821-FP and FP-920 fluorescence detectors (Jasco Benelux B.V., Maarssen, The Netherlands). Separations were achieved on a stainless-steel analytical column (150x4.6 mm internal diameter) packed with Inertsil ODS-80A material (5 µm particle size), delivered by Alltech Applied Science (Breda, The Netherlands). The mobile phase was identical in both assays and was composed of 1.0 M aqueous ammonium acetate (pH 5.5)-water-acetonitrile (10:72.5:17.5, v/v/v) with the pH adjusted to 5.5 (acetic acid). The analytical columns were maintained at 60°C using a model SpH99 column oven (Spark Holland, Meppel, The Netherlands). A detailed composition of the various HPLC systems used for the two assays is provided in Table 1. Peak recording and integration were performed with the Chrom-Card data analysis system (Fisons, Milan, Italy). All calibration curves were fitted by weighted ($1/x$) least-squares linear regression analysis using the peak height ratios of LRT and the IS *versus* the nominal concentrations of the standards.

SampleTreatment for Total LRT in Plasma and Urine

Samples were prepared by addition of 100 µl IS solution [100 ng/ml in 10% perchloric acid-acetonitrile (2:1, v/v)] to 200 µl human plasma, or 40-fold diluted urine in plasma, in a 1.5-ml polypropylene vial (Eppendorf, Hamburg, Germany). Samples were mixed vigorously for 30 min on a multi-tube vortex mixer, followed by centrifugation at 23,000x*g* (5 min) at ambient temperature. A 250-µl volume of the clear supernatant was transferred to a low volume glass insert, from which 200 µl (or 20 µl in case of QC and RC samples containing 750 ng/ml LRT and NX 211, respectively) were injected into the HPLC system.

Table 1.
Composition of the two HPLC systems

Equipment	Sensitive urine	Total plasma/urine
Pump 4100	---	Flow-rate: 1.25 ml/min
Pump 3200	Flow-rate: 0.75 ml/min	---
Autosampler 717p	Yes	Yes
Column oven SpH99	T=60°C	T=60°C
Column ODS-80A	Yes	Yes
Beam Boost	Lamp: λ=254 nm	---
	Coil: 25 m; ID: 0.3 mm	
Detector FP-920	λ_{ex}=378 nm	---
	λ_{em}=420 nm	
	Em band: 40 nm	
Detector 821-FP	---	λ_{ex}=378 nm
		λ_{em}=420 nm
		Em band: 30 nm

Sample Treatment for LRT in the Sensitive Urine Assay

A 100-µl volume of IS solution (50 ng/ml in 25 mM aqueous ammonium acetate, pH 3.0) was added to a 12-ml glass tube supplied with a PTFE-covered screw cap containing 150 µl urine. After incubation for 30 min at room temperature, 0.8 g solid sodium chloride was added, followed by extraction with 2 ml *n*-butanol-diethyl ether (3:4, v/v) by vigorous vortex mixing for 5 min. Subsequently, the sample was centrifuged at 4000x*g* (5 min), followed by collection of 1 ml upper organic layer, which was evaporated to dryness under a gentle stream of nitrogen at 70°C for a period of 45 min. The dried residue was redissolved in 150 µl 25 mM aqueous ammonium acetate (pH 3.0) and transferred to a glass insert. A volume of 50 µl (or 10 µl for the QC sample containing 250 ng/ml LRT) was injected into the HPLC system.

Validation

Validation runs of LRT in plasma and urine and of LRT in the sensitive urine assay included a set of calibration samples assayed in duplicate, and LLQ and QC samples in quintuplicate, and was performed on 4 separate occasions. Precisions were calculated by one-way analysis of variance (ANOVA) for each test concentration, using the run-day as the classification variable. The accuracy of at least 80% of the samples assayed at each concentration should be in the range of 80 to 120%. The within-run (WRP) and between-run precisions (BRP) should be <20% at the concentration of the LLQ and <15% at the

concentrations of the QC samples and the average accuracy (ACC) should be within 85-115% for each concentration, including the LLQ.

The extraction recoveries of LRT and the IS in the assay for total LRT in plasma and urine were calculated by comparing peak heights obtained from a sample containing 25.0 ng/ml LRT in phosphate-buffered saline that was extracted (as described for plasma), to those obtained in extracted QC samples prepared in the biological matrix. The extraction recoveries in the sensitive urine assay were calculated by comparing observed peak heights of the processed urine samples of the calibration curves to peak heights obtained from spiked samples containing 1.00 ng/ml LRT and 10.0 ng/ml IS in 25 mM aqueous ammonium acetate (pH 3.0).

The stability of LRT and the IS in plasma and urine was established (*i*) during 3 consecutive freeze-thaw cycles, in which the samples were put at room temperature for 30 min after thawing, and (*ii*) during an overnight incubation at 37°C. The concentrations used were 20.0 and 75.0 ng/ml for plasma and 250 and 2500 ng/ml for urine, and were analyzed using the assay for the determination of total LRT in plasma and urine.

The selectivity of the assays was tested by the degree of separation of the compounds of interest and possible other chromatographic peaks caused by endogenous components and/or potentially co-administered drugs. The interference from endogenous material for LRT and the IS in human plasma and urine was determined by visual inspection of HPLC profiles of 5 processed blank plasma and urine samples obtained from 5 healthy volunteers. Interference from potentially co-administered drugs was tested at a spiked concentration of 10 μg/ml in a blank plasma extract for the assay of total LRT in plasma and urine, and at 10 μg/ml in 25 mM aqueous ammonium acetate (pH 3.0) for LRT in the sensitive urine assay. The tested compounds included acetaminophen, alizapride, codeine, dexamethasone, domperidon, metoclopramide, morphine, leucovorin, lorazepam, paroxetine, and ranitidine.

Results and Discussion

In approaching the present analytical procedures, we used our own previous RP-HPLC procedure for the quantitative determination of total nonliposomal LRT (lactone plus carboxylate forms) in human whole blood as a starting point [18]. Because of the pH-dependent instability of the α-hydroxy-δ-lactone moiety in the core structure of LRT, resulting in the ring-opened carboxylate from at hight pH and the ring-closed lactone form at low pH, we decided to focuss only on measurement of total concentrations in the present study, since disruption of liposomes while maintaining the physiologic lactone to carboxylate ratio may not be feasible. The choice of the IS, 6,7-dimethoxy-4-methylcoumarin, and the optimal fluorescence wavelength couple of LRT (378/420 nm) was based on earlier work descibed for determination of LRT in human blood and dog plasma by Selinger et al. [19] and Stafford and St. Claire [20], respectively.

For the purpose of assay validation, all QC samples were prepared with the carboxylate form of LRT, to ensure a quantitative conversion to the lactone species of the total amount of LRT, prior to measurement, present in plasma and urine of clinical samples.

Assay of Total LRT in Plasma and Urine

Initially, the assay of total LRT in plasma and urine was validated with only LLQ and QC samples of nonliposomal LRT (free base) in plasma and urine, using an extraction time of 15 min. The calibration curves were linear in the range of 1.00 to 100 ng/ml with Pearson's regression correlation coefficients ranging from 0.9986 to 0.9997, by using weighted ($1/x$) linear least-squares regression analysis. The retention times of LRT and the IS were 11 and 15 min respectively, with an overall run time of 20 min.

One of the tested blank plasma sample specimens showed a minor (unknown) interfering peak in the chromatogram for LRT, and was replaced by a new blank plasma obtained from a healthy volunteer to enable accurate determination of the LLQ. No interfering peaks with retention times around the IS were found in the tested blank plasma samples. In the 5 (40-fold diluted) blank human urine samples, no interfering peaks were found for LRT; however, all tested urine samples showed a small peak with the same retention time as the IS, but this interference did not significantly alter the observed data. The tested drugs potentially co-administered with NX 211 did not interfere with the analytes of interest. Some minor peaks were found with retention times of 1 to 3 minutes, causing no problem for the determination of LRT in plasma and urine.

The LLQ was established in plasma at 1.00 ng/ml and in urine at 100 ng/ml, with 95% of the LLQ samples falling within the acceptable accuracy range of 80 to 120% [17]. The within-run and between–run precisions at the 5 tested concentrations in plasma, including the LLQ, were <7.5% and <4.1%, respectively, with the accuracy ranging from 96 to 110% (Table 2). The within-run and between-run precisions in urine at the 3 tested concentrations were <6.7% and <4.4%, respectively, with an accuracy range of 97 to 108% (Table 2). The extraction recoveries, estimated by comparing peak heights obtained by direct injection of standard solutions containing 25.0 ng/ml LRT and IS in blank plasma extracts to those obtained in extracted plasma samples of the calibration curves, were 89±8.2% (mean ± standard deviation) and 67±4.8% for LRT and the IS, respectively. No loss of LRT was estimated at the tested concentrations after 3 freeze-thaw cycles or during overnight incubation of the samples at 37°C (data not shown).

Table 2.
Validation characteristics of total LRT in plasma and urine.

Matrix	Nom.conc. (ng/ml)	Mean (ng/ml)	WRP[a] (%)	BRP[a] (%)	ACC[a] (%)
Plasma	1.00 [b]	0.959	7.5	4.1	96
	4.00	3.84	7.4	c	96
	20.0	22.0	2.7	1.0	110
	75.0	81.4	2.3	2.1	109
	750	789	2.8	2.4	105
Urine	100 [b]	96.8	6.7	4.4	97
	250	269	3.0	0.12	108
	2500	2525	4.3	2.1	101

a: Abbreviations: WRP, within-run precision; BRP, between-run precision; ACC, average accuracy
b: Lower limit of quantitation sample
c: No additional variation was observed as a result of performing the assay in different runs

Using an extraction time of 15 min, we noted that the extraction recovery of plasma samples containing NX 211 (liposomal LRT) was approximately 10% lower for LRT as compared to plasma samples spiked with nonliposomal LRT (free base). The extraction efficiency of the samples containing NX 211 was eventually increased by extending the vortex-mixing time to 30 min, at which maximum recovery was reached (data not shown). The assays of total LRT in plasma and urine were re-validated during 3 analytical runs (with a 30-min mixing time during sample extraction), and the calibration curves were assayed in duplicate with the 4 QC samples of LRT and 2 RC samples containing NX 211 spiked at concentrations of 20.0 and 750 ng/ml, both in plasma, in triplicate. The Pearson's regression correlation coefficients in the re-validation runs ranged from 0.9995 to 0.9998, and the range of the within-run and between-run precisions of the QC samples containing LRT were 1.8 to 3.6% and 0.73 to 2.5% respectively, with an overall accuracy between 99 and 104%. The within-run precisions of the RC samples were 1.9 and 2.5%, respectively, for the samples containing 20.0 and 750 ng/ml, whereas the respective between-run precisions were 2.3 and 7.3%. The extraction recoveries of LRT in the QC and RC samples containing 20.0 ng/ml LRT and NX 211 were 90±2.1% and 85±3.9%, respectively, and 83±3.0% (LRT) and 82±3.0% (NX 211) for the samples containing 750 ng/ml.

Representative RP-HPLC chromatograms derived from a blank human plasma pool and a plasma sample spiked to contain 10.0 ng/ml LRT (free base) are shown in figs. 2A and 2B.

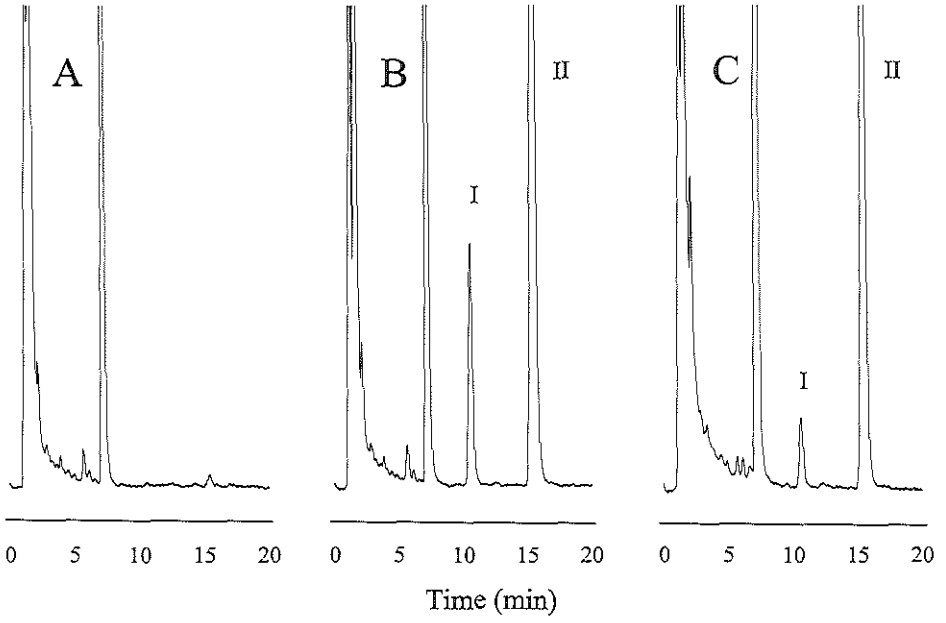


Figure 2. *Chromatograms of a blank human plasma sample (A), a plasma sample spiked with 10.0 ng/ml LRT free base (B) and a plasma sample obtained from a patient 8 h after the administration of NX 211 at a dose level of 0.8 mg (C). Peaks labeled I and II correspond to LRT and the IS, respectively.*

An additional chromatographic peak was found in the RC samples containing NX 211 with a retention time of approximately 48 min. This peak was later identified as a photochemical degradation product of NX 211 by comparison of the compound's chromatographic behavior on the RP-HPLC column and spectroscopic properties with a pure reference standard. Isolation, purification and structural identification, in addition to the role of this compound in the overall drug disposition will be described separately.

Assay for the Sensitive Determination of LRT in Urine

Previous studies have shown that renal clearance of LRT in patients treated with nonliposomal drug was low, with approximately 10-14% of the delivered dose excreted as unchanged parent drug in urine [6]. In order to allow determination of low concentrations of LRT in urine, which can be anticipated following treatment with NX 211 at low dosages, an

assay was also required with increased sensitivity as compared to the assay described for total LRT concentrations in plasma and urine with an LLQ (for urine) of 100 ng/ml (see above). The sensitivity of this assay could theoretically be improved by decreasing the dilution factor used for urine samples prior to extraction. However, this will likely result in substantially prolonged run times in order to get sufficient separation between the peaks of interest (i.e. LRT and the IS) and those resulting from the presence of endogenous material, which would in turn compromise assay sensitivity.

Eventually, the assay sensitivity could be significantly improved, in part, by increasing the fluorescence intensity of LRT through a modification of the detection procedure. LRT is known to be slightly light sensitive [19] and since photochemical reactor units in combination with HPLC has been described for a wide variety of other compounds [21-25], where increased detector signal outputs have been described from 2 to 80 folds, we have evaluated the impact of post-column photodegradation on the fluorescence activity of LRT. Post-column exposure of LRT to UV light (254 nm) results in a loss of the piperazinomethylene moiety on C7 of the LRT molecule, as determined by electro-spray ion-trap mass spectrometry [m/z=409 (LRT-C7 side chain)]. The influence of the photochemical reaction unit on the fluorescence of LRT was estimated by injections of 50 µl of 5 ng/ml LRT in 25 mM ammonium acetate (pH 3.0) onto the HPLC system as described for this assay. The flow rate was varied from 0.50 to 2.00 ml/min, resulting in irradiation times of 300 to 75 s. At each flow rate, 2 injections were performed, one with the lamp of the photochemical reaction unit on and another one with the lamp switched off. The ratios of the peak heights obtained with the lamp on and off were calculated. As displayed in fig. 3, the fluorescence intensity of LRT increased 9 to 15-fold depending on the flow rate used (0.50 to 2.00 ml/min). The use of a flow rate set at 0.75 ml/min (i.e. and irradiation time of 200 s) resulted in a 14-fold increased fluorescence signal of LRT, and was associated with an acceptable total run time (35 min) with retention times of 19 and 24 min for LRT and the IS, respectively.

Fig. 4 shows representative chromatograms of a blank human urine sample and a sample spiked with 2.50 ng/ml LRT (free base). The calibration curves of LRT were linear in the range of 0.500 to 10.0 ng/ml, with Pearson's correlation coefficients ranging from 0.9954 to 0.9994, also using weighted (1/x) least-squares linear regression analysis. No analytical interference was found between LRT or the IS and of the tested drugs potentially co-administered with NX 211. However, a number of three additional peaks were found with retention times of 6, 7 and 10 minutes. Since these elute in the big front of the chromatograms of urine and high concentrations of the drugs were spiked these peaks have no impact on the determination of low concentration of LRT in urine.

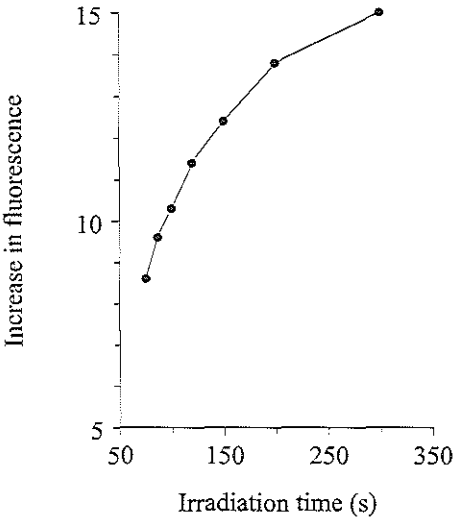


Figure 3. *Influence of the photochemical reaction unit on the fluorescence intensity of LRT.*

Small peaks with the same retention time as LRT and the IS were found in all of the tested blank urine samples, so the LLQ could not be established below a concentration of 0.500 ng/ml, with 80% of all samples in the acceptable range of accuracy. The within-run and between-run precisions at the 4 tested concentrations were <5.5 and <10%, respectively, with the accuracy ranging from 97 to 108% (Table 3). Taking into consideration that only approximately half of the added volume of the organic layer was evaporated, the extraction recoveries were around 80 and 92% for LRT and IS respectively.

Assay Application

The suitability of the developed methods for clinical use was demonstrated by the determination of LRT in biological specimens obtained from a patient treated with NX 211 at a dose of 0.8 mg. Examples of the patient's sample trace are shown in Fig. 2C (plasma) and Fig. 4C (urine). Distinct peaks were obtained for LRT in both matrices that were well resolved from endogenous components.

Table 3.

Validation characteristics of total LRT urine (sensitive assay).

Nom.conc. (ng/ml)	Mean (ng/ml)	WRP[a] (%)	BRP[a] (%)	ACC[a] (%)
0.500 [b]	0.539	5.5	c	108
1.25	1.31	3.0	3.1	105
7.50	7.27	2.8	6.7	97
250	243	4.2	10	97

a: Abbreviations: WRP, within-run precision; BRP, between-run precision; ACC, average accuracy

b: Lower limit of quantitation sample

c: No additional variation was observed as a result of performing the assay in different runs

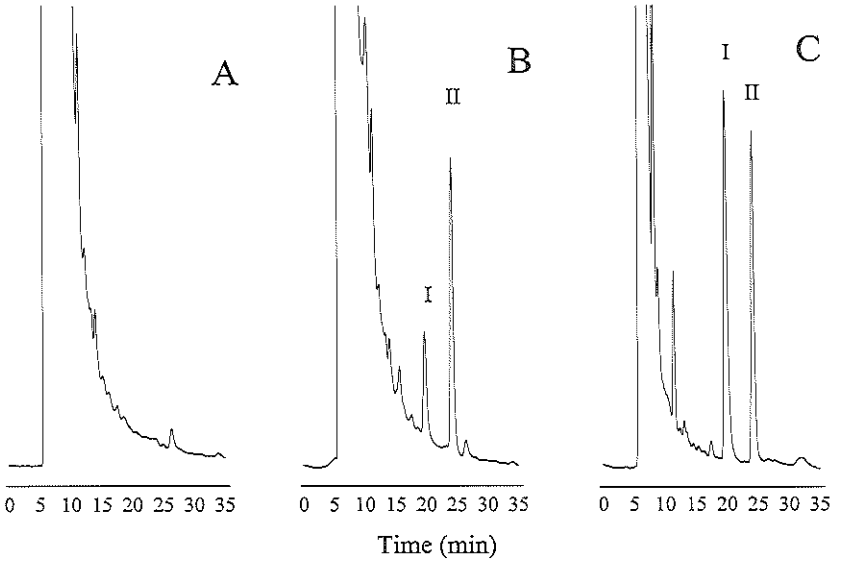


Figure 4. *Chromatograms of a blank human urine sample (A), a urine sample spiked with 2.50 ng/ml LRT free base (B) and a urine sample obtained from a patient, collected 12-24 h after the administration of NX 211 at a dose level of 0.8 mg (C). Peaks labeled I and II correspond to LRT and the IS, respectively.*

Conclusion

In conclusion, we have developed and evaluated new liquid chromatographic methods for measuring total LRT levels in human plasma and urine. The primary elements of novelty described in this work are the use of human plasma as biological matrix containing liposomal LRT (NX 211) and the utilization of a photochemical reaction unit to increase the native fluorescence intensity of LRT in order to achieve sub-nanogram per milliliter determinations. The methods were shown to meet the current requirements as to validation of bioanalytical methodologies [17], providing excellent precision and accuracy. The described methods permit the analysis of patient samples, and will be implemented in an ongoing clinical trial to investigate the disposition of LRT in cancer patients receiving NX 211.

References

1 Wall ME, Wani MC, Cook CE, et al. J Am Chem Soc 88: 3888, 1966.

2 Takimoto CH, Arbuck SG. in Chabner BA, Longo DL (Editors), Cancer chemotherapy and Biotherapy, Lippencott-Raven Publishers, 463, 1996.

3. Luzzio MJ, Besterman JM, Emerson DL, et al. Synthesis and antitumor activity of novel water soluble derivatives of camptothecin as specific inhibitors of topoisomerase I. J Med Chem. 1995;38:395-401.

4. Emerson DL, Besterman JM, Brown HR, et al. In vivo antitumor activity of two new seven-substituted water-soluble camptothecin analogues. Cancer Res. 1995;55:603-609.

5. Besterman JM. Topoisomerase I inhibition by the camptothecin analog GI147211C. From the laboratory to the clinic. Ann N Y Acad Sci. 1996; 803:202-209.

6. Gerrits CJ, Creemers GJ, Schellens JH, et al. Phase I and pharmacological study of the new topoisomerase I inhibitor GI147211, using a daily x 5 intravenous administration. Br J Cancer. 1996;73:744-750.

7. Eckhardt SG, Baker SD, Eckardt JR, et al. Phase I and pharmacokinetic study of GI147211, a water-soluble camptothecin analogue, administered for five consecutive days every three weeks. Clin Cancer Res. 1998;4:595-604.

8. Paz-Ares L, Kunka R, DeMaria D, et al. A phase I clinical and pharmacokinetic study of the new topoisomerase inhibitor GI147211 given as a 72-h continuous infusion. Br J Cancer. 1998;78:1329-1336.

9. Hertzberg RP, Caranfa MJ, Holden KG, et al. Modification of the hydroxy lactone ring of camptothecin: inhibition of mammalian topoisomerase I and biological activity. J Med Chem. 1989;32:715-720.

10. Burke TG, Gao X. Stabilization of topotecan in low pH liposomes composed of distearoylphosphatidylcholine. J Pharm Sci. 1994;83:967-969.

11. Subramanian D, Muller MT. Liposomal encapsulation increases the activity of the topoisomerase I inhibitor topotecan. Oncol Res. 1995;7:461-469.

12. Daoud SS, Fetouh MI, Giovanella BC. Antitumor effect of liposome-incorporated camptothecin in human malignant xenografts. Anticancer Drugs. 1995;6:83-93.

13. Jones CB, Clements MK, Wasi S, et al. Sensitivity to camptothecin of human breast carcinoma and normal endothelial cells. Cancer Chemother Pharmacol. 1997;40:475-483.

14. Lundberg BB. Biologically active camptothecin derivatives for incorporation into liposome bilayers and lipid emulsions. Anticancer Drug Des. 1998;13:453-461.

15. Sadzuka Y, Hirotsu S, Hirota S. Effect of liposomalization on the antitumor activity, side-effects and tissue distribution of CPT-11. Cancer Lett. 1998;15:99-106.
16 Emerson DL, Amirgahari N, Bendele R, et al. NX-211, a liposomal formulation of Lurtotecan demonstrates enhanced pharmacokinetic and antitumor activity. Proc AACR 39: 278, 1998.
17 Shah VP, Midha KK, Dighe S, et al. J Pharm Sci 81: 309, 1992.
18 Sparreboom A, Loos WJ, Schellens JHM, et al. Improved analytical methods for the determination of camptothecin analogues in patient samples. Proc AACR 38: 306, 1997.
19. Selinger K, Smith G, Depee S, et al. Determination of GI147211 in human blood by HPLC with fluorescence detection. J Pharm Biomed Anal. 1995;13:1521-1530.
20. Stafford CG, St Claire RL 3rd. High-performance liquid chromatographic analysis of the lactone and carboxylate forms of a topoisomerase I inhibitor (the antitumor drug GI147211) in plasma. J Chromatogr B Biomed Appl. 1995;663:119-126.
21. Scholten AH, Welling PL, Brinkman UA, et al. Use of PTFE coils in post-column photochemical reactors for liquid chromatography--application to pharmaceuticals. J Chromatogr. 1980;199:239-248.
22. Salamoun J, Frantisek J. Determination of methotrexate and its metabolites 7-hydroxymethotrexate and 2,4-diamino-N10-methylpteroic acid in biological fluids by liquid chromatography with fluorimetric detection. J Chromatogr. 1986;378:173-181.
23. Schaefer HG. Determination of BAY y 3118, a novel 4-quinolone, in biological fluids using high-performance liquid chromatography and photothermal post-column derivatization. J Chromatogr. 1993;616:87-93.
24. Di Pietra AM, Andrisano V, Gotti R, et al. On-line post-column photochemical derivatization in liquid chromatographic-diode-array detection analysis of binary drug mixtures. J Pharm Biomed Anal. 1996;14:1191-1199.
25. Tse SY, Whetsel R. Determination of minalrestat (an aldose reductase inhibitor) in rat, dog and human plasma by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 1998;709:127-135.

Chapter 3

Phase I and Pharmacologic Study of Liposomal Lurtotecan (NX 211): Urinary Excretion Predicts Hematologic Toxicity

D.F.S. Kehrer, A.M. Bos, J. Verweij, H.J. Groen, W.J. Loos, A. Sparreboom, M.J.A. de Jonge, M. Hamilton, T. Cameron, E.G.E. de Vries

Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam, the Netherlands; Department of Medical Oncology, University Hospital Groningen, the Netherlands; and Gilead Sciences Incorporation, Boulder, CO, USA.

Submitted

Abstract

Purpose: To determine the maximum-tolerated and recommended dose, toxicity profile and pharmacokinetics of the liposomal topoisomerase I inhibitor, lurtotecan (NX 211) administered as a 30-minute IV infusion once every 3 weeks in cancer patients.

Patients and Methods: NX211 was given by peripheral infusion. Dose escalation decisions were based upon all toxicities during the first cycle as well as pharmacokinetic parameters. Serial plasma, whole blood, and urine samples were collected for up to 96 hours following the end of infusion and drug levels were determined by high-performance liquid chromatography.

Results: Twenty-nine patients (13 males/16 females; median age, 56 years; range, 39-74) received 77 courses of NX 211 at dose levels of 0.4 (n=3 patients), 0.8 (6), 1.6 (3), 3.2 (6), 3.8 (6) and 4.3 mg/m^2 (5). Neutropenia and thrombocytopenia were the dose-limiting toxicities, and were not cumulative. Other toxicities, including nausea, vomiting and fatigue, were mild to moderate. Nine patients had stable disease (some of which prolonged) while on treatment, whereas one of these with an adenocarcinoma of unknown origin (ACUP) had a significant regression just not reaching the criteria of partial response (PR). The systemic clearance of lurtotecan in plasma and whole blood was 0.82±0.78 and 1.15±0.96 L/h/m^2, respectively. Urinary recovery (Fu) of lurtotecan was 10.1±4.05% (range, 4.9 to 18.9). In contrast to systemic exposure measures, the dose excreted in urine (i.e., dose×Fu) was significantly related to the percent decrease in neutrophil and platelet count at nadir (P<.00001).

Conclusion: The dose-limiting toxicities of NX 211 are neutropenia and thrombocytopenia. The recommended dose for phase II studies is 3.8 mg/m^2 once every three weeks. Pharmacologic data suggest a relationship between exposure to lurtotecan and NX 211-induced clinical effects.

Introduction

Lurtotecan (7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin, also known as GI147211 (Fig. 1), is a semisynthetic analogue of camptothecin, a cytotoxic plant alkaloid that was first extracted from Camptotheca acuminata [1]. Structurally, lurtotecan is unique among camptothecin drugs, because of a dioxalane moiety on the A-ring and a bulky 4-methyl-piperazinomethylene group on the C-7 position. These molecular modifications have resulted in enhanced aqueous solubility as compared to the original agent and increased affinity of the drug for DNA topoisomerase I, the cellular locus through which camptothecin analogues produce their antitumor activity. Its mechanism of action is based on stabilization of the cleavable complex formed by the intranuclear enzyme topoisomerase I and DNA, and on induction of the single-stranded DNA-breaks [2]. The cytotoxicity of the topoisomerase I inhibitors is distinctly S-phase specific, and various preclinical studies with

lurtotecan and other camptothecin drugs have suggested that prolonged exposure, achieved either by repeated doses or prolonged infusion, might be beneficial for efficacy profiles.3 Preclinical in vivo studies with lurtotecan as a single agent demonstrated that it is an effective inhibitor of mammalian DNA topoisomerase I, with at least similar potency as the related agent, topotecan [1,4].

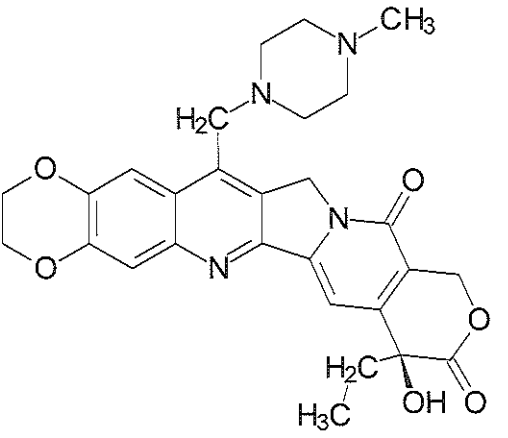


Figure 1. *Chemical structure of lurtotecan.*

Several clinical trials with lurtotecan have been performed to exploit the possible schedule dependence, focussing on intermittent (daily for 5 days every 3 weeks) [5-8] or prolonged IV administration (72-hour infusion every 4 weeks, and 7, 14 or 21-day infusion schedules) [9-10]. These studies have shown that the pharmacokinetic behavior of lurtotecan, in analogy to other topoisomerase I inhibitors, is significantly influenced by a chemical pH-dependent hydrolysis of the active lactone form to the ring-opened carboxylate form, which is devoid of biological activity [11]. Based on this knowledge, considerable effort has recently been put in the development of alternative pharmaceutical vehicles that would allow prolonged systemic exposure to the biologically active lactone form. Among various approaches, liposomal encapsulation of camptothecin analogues was shown to effectively diminish lactone hydrolysis [12-18]. Besides this, liposomal encapsulated anticancer drugs have been studied extensively both in the laboratory and in the clinic, with reports of prolonged plasma exposure, improved tumor delivery, decreased systemic toxicity and increased efficacy for a variety of cytotoxic drugs [19]. Increased antitumor activity by enhancement of tissue distribution and systemic drug availability of liposome-encapsulated topoisomerase I inhibitors has been found in rodent models, including improved therapeutic

efficacy for a new stable unilamellar liposomal formulation of lurtotecan (NX 211) as compared to non-liposomal lurtotecan [20].

In view of the above, a phase I open-label, dose-escalating trial was initiated to investigate the clinical utility of NX 211 administration. The objectives of this study were *i*) to assess the safety and toxicity profile of this lurtotecan formulation; *ii*) to determine the Dose Limiting Toxicities (DLT), the Maximum-Tolerated Dose (MTD) as well as the recommended dose for phase II studies with the drug given by IV administration once every 3 weeks; and *iii*) to examine the disposition of this drug.

Patients and Methods

Eligibility Criteria

Patients were eligible if they had a histologically confirmed diagnosis of advanced solid tumor refractory to standard chemotherapy or a malignancy for which there was no effective standard chemotherapeutic regimen. Additional criteria included: age ≥ 18 years; Eastern Cooperative Oncology Group (ECOG) status of 0-2; no previous treatment with antineoplastic agents for at least 3 weeks (4 weeks in case of carboplatin or an investigational drug, 6 weeks in case of nitrosoureas or mitomycin C), or radiotherapy exceeding 25% of the bone marrow volume; adequate bone marrow function defined as absolute neutrophil count (ANC) ≥ 1.5x109/L and platelets ≥ 100x109/L, adequate renal function as defined by a serum creatinine within normal limits; adequate liver function as defined by bilirubin within normal limits, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ≤ 2.5 times upper limit of normal in the absence of liver metastasis and ≤ 5 with documented liver metastasis; and no known hypersensitivity to systemic liposomal formulations or any drug chemically related to lurtotecan. The current clinical protocol was approved by the Rotterdam Cancer Institute Ethics Board, and the Ethics Board of the University Hospital Groningen and all patients signed a written informed consent before study entry.

On-Study and follow-up Investigations

Patients underwent a complete physical examination at enrollment and at the start of every new NX 211 course. At these visits, ECOG performance status and weight were recorded. Determination of hematologic parameters (i.e., full blood count with white blood cell differential) was performed twice weekly at least during the first two cycles, then weekly for following cycles. Clinical chemistry analyses (i.e., electrolytes, creatinine, calcium, random glucose, albumin, phosphate, urea, uric acid, total protein, triglycerides, total cholesterol, bilirubin, alkaline phosphatase, ALAT, ASAT and lactate dehydrogenase) were performed weekly. ECG was performed before study entry, on completion of the study and during treatment only if clinically indicated. Tumors were assessed radiologically before patients were enrolled on the study, and after every even-numbered course.

Pharmaceutical Preparation and Drug Administration

NX 211 was formulated as a sterile liposomal dispersion in a buffer composed of 10-mM ammonium chloride and 9% sucrose, and was obtained from Gilead Sciences Inc. (San Dimas, CA). Detailed description on the liposomal preparation of the unilamellar small liposomes composed from hydrogenated soy phosphatidylcholine and cholesterol has recently been published [20]. The drug product was supplied in 50-mL vials containing 5 mg of lurtotecan. Each vial contained 80 mg of hydrogenated-soy phosphatidylcholine, 20 mg of cholesterol, 0.9 g of sucrose, 2 mg of citric acid and 5 mg of ammonium chloride to a total volume of 10 mL. Dose solutions for administration were prepared under aseptic conditions by dilution of the pharmaceutical product with sterile 5% dextrose to a volume of 25 mL for administration by a controlled-rate pump. The whole process described above was performed under strict light protection.

NX 211 was administered on day 1 of each 21-day course as a 30-minute IV infusion under complete light protection, where both the syringe and infusion line were totally wrapped in aluminum foil. All patients were admitted to the hospital for the first dose of the drug to facilitate pharmacokinetic sampling (see below). Subsequent doses were given in an outpatient setting. Chemotherapy was repeated every 3 weeks for at least 2 courses. No standard premedication was given in any course.

Study Design

The starting dose of NX 211 (0.4 mg/m^2) was the equivalent of 1/50 of the acute LD10 in mice. This safety margin, as compared to the classical starting dose of 1/10 of the LD10 in mice, is implemented based on data from the parent compound and topotecan. Dose escalation was performed based on toxicity and pharmacokinetics. For safety, the next dose level was not opened until at least 3 patients were assessable in the first course. In case only one patient developed dose-limiting toxicity (DLT), the dose level was expanded with another 3 patients to a total of 6. In case less than 2 of 6 patients experienced DLT, or no DLT was observed, the dose level for the next patient cohort was established based on both clinical toxicity and pharmacokinetic data observed at the previous dose level. In addition information gathered from two other parallel phase I studies with NX 211 (day 1 ,2 ,3 q 3 weeks and day 1 and 8 q 3 weeks) was also taken into account.

Toxicity Evaluation

Toxicity was evaluated and graded according to the NCI Common Toxicity Criteria version 2.0 (CTC). Hematologic DLT was defined as ANC $< 0.5 \times 109/L$ for ≥ 7 days and/or associated with fever, and/or platelets $\leq 25 \times 109/L$ or bleeding episodes requiring platelet transfusion. Non-hematologic DLT was defined as any toxicity CTC grade ≥ 3, with the exception of vomiting in the absence of appropriate antiemetic therapy, as well as CTC grade ≥ 2 neurotoxicity or cardiac toxicity. In case DLT was reached in ≥ 2 of 3 or ≥ 2 of 6 patients,

dose escalation was ceased, and the dose level defined as maximum tolerable dose (MTD). Once the MTD had been determined, intermediate dose levels could be studied. The recommended dose was defined as one dose level below the MTD.

Response Evaluation

Tumor response definitions were based on the WHO criteria (WHO handbook for reporting results of cancer treatment, WHO, Geneva, 1979).

Pharmacokinetic Sample Collection and Preparation

Blood samples for pharmacokinetic analysis were drawn from a vein in the arm opposite to that used for drug infusion, and collected in 7-mL glass tubes containing lithium heparin as anticoagulant. Duplicate samples were obtained before drug administration and at 0.5 (end of infusion), 1, 1.5, 2.5, 4, 6, 8, 24, 48, 72 and 96 hours after start of infusion. At each sampling time point, one aliquot of whole blood was immediately transferred to a polypropylene vial and stored at –80°C, while another was processed to plasma by centrifugation for 5 minutes at 3000xg (4°C), which was then also stored at –80°C until the time of analysis. Complete urine collections were obtained for the duration of the study in 12-hour or 24-hour portions, and aliquots were stored frozen in polypropylene vials. In addition to the protocol, in those patients who consented, complete collections of feces up to 96 hours were also obtained in polystyrene containers, and stored immediately at –80°C. After thawing, these samples were homogenized individually in 4 volumes of phosphate-buffered saline using an Ultra-Turrax T25 homogenizer (IKA-Labortechnik, Dottingen, Germany).

Analytical Methods

Lurtotecan dihydrochloride monohydrate (lot: U2044/164/1, containing 78.11% of the free base) was supplied by Gilead Sciences Inc. (San Dimas, CA) and was used as a reference standard for all reversed-phase high-performance liquid chromatographic (HPLC) assays. Plasma and urine concentrations of lurtotecan were determined using validated HPLC assays as described previously [21].

For the determination of lurtotecan in whole blood and feces, the assay for total plasma concentrations was modified as outlined below. The HPLC systems consisted of a constaMetric 3200 solvent delivery system (LDC Analytical, Riviera Beach, FL), a Waters 717plus autosampler (Milford, MA), an Inertsil-ODS 80A analytical column (150×4.6 mm I.D., 5 μm particle size; Alltech Applied Sciences, Breda, the Netherlands) maintained at 60°C by a model SpH99 column oven (Spark Holland, Meppel, the Netherlands), a Beam Boost photochemical reactor unit supplied with a coil of 25 m×0.3 mm I.D. (ICT-ASS-Chem, Bad Homburg, Germany), and a Jasco FP-920 fluorescence detector (Jasco, Maarssen, the Netherlands) operating at excitation and emission wavelengths of 378 and 420 nm (40 nm

band width), respectively. The mobile phases consisted of 1 M aqueous ammonium acetate-water-acetonitrile (100:725:175, v/v/v), with the pH adjusted to 5.5 with acetic acid. The flow rates were set at 1.25 and 0.75 mL/min for the determination of total lurtotecan levels, i.e. lurtotecan in and out-side the liposomes, in blood and feces samples, respectively.

Aliquots (50 µL) of heparinized whole blood were pretreated with 500 µL of 5% (w/v) aqueous perchloric acid-acetonitrile (5:1, v/v) in 1.5-mL polypropylene tubes (Eppendorf, Hamburg, Germany). The samples were vigorously vortex-mixed for 30 minutes on a multi-tube vortex mixer, followed by centrifugation for 5 minutes at 23,000xg at ambient temperature. A volume of 250 µL of the clear supernatant was transferred to a low volume insert of glass, from which 200 µL were injected into the HPLC system. The calibration curves were constructed in saline at concentrations of 0.25, 0.50, 1.00, 5.00, 10.0 and 25.0 ng/mL, by serial dilutions of a lurtotecan working solution containing 0.10 mg/mL (expressed as free base). Three pools of quality-control samples were prepared in heparinized whole blood at concentrations of 0.40, 20.0 and 2000 ng/mL, by addition of appropriate volumes of lurtotecan in saline to whole blood. In addition, to minimize potential differences with clinical samples, a lurtotecan recovery-control sample containing 7.50 ng/mL (as NX 211), was also analyzed simultaneously. The sample containing 2000 ng/mL was diluted 100-fold in phosphate-buffered saline prior to extraction.

Aliquots (100 µL) of feces homogenate were deproteinized and acidified with 1000 µL of 5% (w/v) aqueous perchloric acid-acetonitrile (5:1, v/v) containing 6,7-dimethoxy-4-methylcoumarin at a concentration of 100 ng/mL (Sigma, St. Louis, MO), which was used as the internal standard. Subsequently, the samples were vigorously vortex-mixed for 15 minutes on a multi-tube vortex mixer, followed by centrifugation at ambient temperature at 23,000×g for 5 minutes. A 100-µL volume of supernatant was transferred to a limited volume insert of glass, from which 10 µL were injected onto the analytical column. Spiked homogenized fecal samples used as calibration standards in concentrations of 10, 25, 50, 100 and 250 ng/mL were prepared by addition of 10 µL of serial dilutions in saline from the lurtotecan working solution to 240 µL of drug-free feces homogenates. Three pools of quality-control samples containing lurtotecan at 40, 200 and 2000 ng/mL were prepared by addition of appropriate volumes of lurtotecan in saline to blank human feces homogenates. The sample containing 2000 ng/mL was diluted 10-fold in a mixture of saline and the extraction solution (1:10, v/v) prior to injection.

Validation of both assays included a set of calibration samples assayed in duplicate, with all samples in quintuplicate, and was performed on 4 separate occasions. Within-run and between-run precision, calculated by one-way analysis of variance for each concentration using the run-day as classification variable, ranged between 2.9-13.2% and 3.9-12.4%, respectively, whereas accuracy of both assays was between 94.9-106%. The mean extraction recoveries for lurtotecan in feces specimens and whole blood were between 97-103%.

Pharmacologic Data Analysis

Individual plasma and whole blood concentrations of lurtotecan were fit to a model with multi-exponential functions by the Siphar version 4.0 software package (InnaPhase Co., Philadelphia, PA), using Powell's method. Model discrimination was assessed by a variety of considerations, including visual inspection of the predicted curves, dispersion of residuals, minimization of the sum of weighted squares residuals, and the Akaike and Schwarz criteria. In all cases, concentration-time profiles were best fit to a mono-exponential equation after zero-order input with weighting according to y_{obs}^{-1}. Final values of the iterated parameters of the best-fit equation were used to calculate pharmacokinetic parameters using standard equations. The disposition half-life was calculated as $\ln 2/k$, in which k is the elimination rate constant in h^{-1}. The total plasma clearance of lurtotecan was calculated by dividing the dose (expressed in mg base equivalents per squared meter of body surface area) by the observed area under the plasma concentration versus time curve extrapolated to infinity (AUC). The volume of distribution at steady state was calculated using the same program. The fraction of the absolute NX 211 dose administered to patients excreted in feces and urine (Fu) as unchanged lurtotecan was expressed as a percentage.

Relationships between various exposure measures (e.g. plasma AUC) and hematologic toxicity were evaluated by sigmoid maximum-effect models using Siphar. Hematological pharmacodynamics were evaluated by analysis of the absolute nadir values of blood cell counts or the relative hematologic toxicity, i.e., the percent decrease in blood cell count, which was defined as:

$$\% \text{ decrease} = [(\text{pretherapy value} - \text{nadir value}) / (\text{pretherapy value})] \times 100\%$$

Within each patient, myelosuppression was described either using continuous variable, consisting of the percent decrease in WBC, ANC and platelet count, or as discrete variable in case of NCI-CTC myelotoxicity grade. Data were fitted to a sigmoid maximum-effect model based on the modified Hill equation, as follows:

$$E = E_0 + E_{max} \times [(KP^{\gamma}) / (KP^{\gamma} + KP_{50}^{\gamma})]$$

In this equation, E_0 is the minimum reduction possible, E_{max} is the maximum response (fixed to a value of 100), KP is the pharmacokinetic parameter of interest, KP_{50} the value of the pharmacokinetic parameter predicted to result in half of the maximum response, and γ is the Hill constant, which describes the sigmoidicity of the curve. Models were evaluated for goodness of fit by minimization of sums of the squared residuals and by reduction of the estimated coefficient of variation for fitted parameters. Significance of the relationships was assessed by construction of contingency tables with subsequent χ^2 analysis.

Statistical Evaluation for the pharmacology data

Parameters of all pharmacologic analyses are reported as mean value ± SD, unless stated otherwise. The relationships between peak plasma concentrations of lurtotecan and the administered level or corresponding AUC values were analyzed by means of Spearman's or Pearson's correlation coefficient, respectively, and linear regression analysis. The difference in pharmacokinetic parameters between patient cohorts was evaluated statistically using the Kruskal-Wallis statistic followed by a Dunn's test, if required, to determine which group differed. Interpatient differences in kinetics were assessed by the coefficient of variation (CV), expressed as the ratio of the SD and the observed mean. Probability values of less than 0.05 were regarded as statistically significant. All statistical calculations were performed using the Number Cruncher Statistical System (NCSS) version 5.X software package (Jerry Hintze, East Kaysville, UT; 1992).

Table 1.
Patient Characteristics

Characteristic	Number of patients
Entered/Evaluable	29
Assessable for Response	27
Age (years)	
Median	56
Range	39-74
Sex	
Male	13
Female	16
Performance score	
0	11
1	17
2	1
Tumor type	
GI*	9
NSCLC**	6
Ovary	1
Miscellaneous	13
Prior chemotherapy	
none	5
1	12
2	5
3(+)	7

* colon, gastric, esophagus; ** non-small cell lung cancer

Results

Patients and Treatment

Twenty-nine eligible patients with advanced carcinomas were recruited into the study. Patient demographic data are listed in Table 1. Two patients were not considered assessable for the response analysis, leaving 27 patients assessable for response, which included 3 patients who received only one dose of NX 211 because of early progressive disease.

Six dose levels of NX 211 (0.4, 0.8, 1.6, 3.2, 3.8 and 4.3 mg/m^2, respectively) were explored during the trial. The 29 patients received a total of 77 courses of NX 211 (median 2, range 1-9). Dose reduction was only required at the highest dose level, where one dose in a single patient was reduced from 4.3 to 3.2 mg/m^2 because of febrile neutropenia in the preceding course. One patient died while on study due to progression of an endometrial sarcoma. There were no drug related deaths. In 3 of 29 patients, scheduled doses had to be delayed for one week, one because of an intercurrent possible pulmonary embolism and three because of hematological toxicity.

Table 2.
Worst Hematologic Toxicity Per Patient (Course 1)

Dose (mg/m^2)	*n*	No. courses	Neutrocytopenia*				Thrombocytopenia*			
			1	2	3	4	1	2	3	4
0.4	3	6	0	0	0	0	0	0	0	0
0.8	6	14	0	0	0	0	1	0	0	0
1.6	3	8	0	0	0	0	1	0	0	0
3.2	6	15	1	1	1	0	0	1	0	0
3.8	6	18	0	0	0	0	2	0	0	0
4.3	5	16**	0	0	0	4	1	1	2	0

* Graded according to NCI-CTC

Decisions on dose escalation were based on toxicity observed in course 1 only (Table 2). At the dose levels (0.4 and 0.8 mg/m^2) no relevant toxicity was observed, but for one patient in the latter group who developed a seizure. Because of a possible relationship to NX 211 this dose level was expanded to six patients. Retrospectively this event however was not considered related to the drug but due to brain metastasis progression. At the next two dose levels (1.6 and 3.2 mg/m^2) hematological toxicity never exceeded grade 2 without remarkable non hematological toxicity. The first two patients at the highest dose level (4.3 mg/m^2), developed dose limiting hematologic toxicity, consisting of grade 4 neutropenia and up to

grade 3 thrombocytopenia. According to the protocol, the previous level (3.2 mg/m^2) was expanded without remarkable toxicity observed. It therefore was decided to add a new dose level of 3.8 mg/m^2 in between the latter two. At this dose level 6 patients were included who received 18 courses (median 3, range 1-6) of NX 211, with thrombocytopenia grade 1 being the most severe observed toxicity. Meanwhile we could not find a pharmacodynamic-pharmacologic relationship nor any other plausible explanation for the steep toxicity difference in dose levels, and discussion arose whether the observed toxicity in the two patients at the highest dose levels could be accidental. It was therefore decided to add further patients at the 4.3 mg/m^2 dose level. One patient treated at this dose level developed grade 4 neutropenia with fever and grade 3 thrombocytopenia in the first course. In view of these data, it was definitely concluded that the 4.3 mg/m^2 dose level was the MTD, and the recommended dose level for phase II studies 3.8 mg/m^2.

Table 3.
Worst Hematologic Toxicity Per Patient (All Courses)

Dose	*n*	No. courses	Neutrocytopenia*				Thrombocytopenia*			
(mg/m^2)			1	2	3	4	1	2	3	4
0.4	3	6	0	0	0	0	0	0	0	0
0.8	6	14	0	0	0	0	1	0	0	0
1.6	3	8	0	1	0	0	1	0	0	0
3.2	6	15	0	2	1	0	0	1	0	0
3.8	6	18	0	0	0	0	4	0	0	0
4.3	5	16**	0	0	1	4	1	1	3	0

* Graded according to NCI-CTC

** in 2 of 16 cycles the patient received a reduced dose of 3.2 mg/m^2

Toxicity Profiles

The main side effects of NX 211 were hematologic, neutropenia and thrombocytopenia being DLT. As can be seen from the data for all courses (Table 3) there was no cumulative hematological toxicity. Grade 3 or 4 neutropenia was observed in 9 of 77 courses, all of them at the highest dose level. Febrile neutropenia was observed in only one patient at the highest (4.3 mg/m^2) dose level tested. Patients developing severe neutropenia also developed the severest thrombocytopenia. Three patients developed grade 3 thrombocytopenia not complicated by bleeding, and not requiring platelet infusion. At the recommended dose level

for phase II studies (i.e., 3.8 mg/m^2), no grade 3 or 4 toxicity was observed, and a 100% dose intensity could be achieved.

Non-hematologic toxicity in this study was not dose limiting, and although some grade three toxicities were observed, no cumulative non-hematological toxicity was found (Table 4). Asthenia was the most common side effect observed, while no grade 3 or 4 nausea and vomiting was seen. One patient at the first cohort experienced chest tightness and a sensation like shortness of breath during the infusion and there were three cases of flushing, symptoms commonly associated with a liposomal infusion-related reaction. After discontinuation of the infusion, the sensation disappeared spontaneously and quickly. Since no serious symptoms persisted, no medication like antihistamines or corticosteroids needed to be administered. At rechallange the infusion was started at half speed, after which no recurrence of the reaction was noted. Other toxicities, as listed in Table 4, were observed sporadically. Overall, NX 211 was very well tolerated in this phase I study.

Table 4.

Related Non-Hematologic Toxicity by Worst Grade Per Patient on Study (*n*=29)

Number (%) of dosed patients with any related adverse event reported in 10+% of all patients

Toxicity	Grade				All	Grade 3 or 4
	1	2	3	4	(%)	(%)
asthenia	6	5	2	0	45	7
fatigue	0	3	0	0	10	0
malaise	2	1	0	0	10	0
headache	2	1	0	0	10	0
nausea	10	5	0	0	52	0
vomiting	8	3	0	0	38	0
anorexia	6	3	0	0	31	0
diarrhea	3	0	0	0	10	0
abdominal pain	4	0	0	0	14	0
alopecia	4	0	0	0	14	0
hypertensitivity reaction	4	0	0	0	10	0

Antitumor Activity

Objective responses were not observed, but 9 patients (NSCLC(3), ACUP(2), ovary, bladder, biliary and leiomyosarcoma) had stable disease (range 2-9 courses) while on treatment with NX 211. There was no apparent relationship between the level of pretreatment and the chance of achieving SD. One patient (ACUP) with stable disease who was treated at the 4.3 mg/m2 dose level, experienced a tumor regression of 49% after six courses with NX 211, but eventually developed progressive disease in the ninth course.

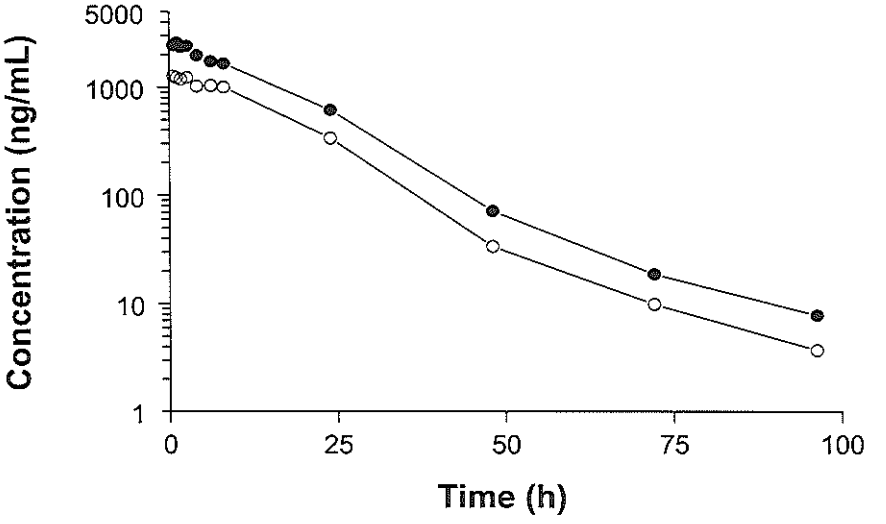


Figure 2. *Representative concentration versus time profiles of lurtotecan in plasma (closed circles) and whole blood (open circles) in a single patient after administration of NX 211 at a dose level of 3.8 mg/m^2.*

Plasma and Blood Disposition

Complete plasma pharmacokinetics were performed in all 29 patients, whereas whole blood data were available for 27 patients. The reported lurtotecan concentration after treatment with NX 211 are the sum of both encapsulated and non-encapsulated drug [21]. A typical example of a plasma concentration-time profile of lurtotecan is shown in Fig. 2. The concentration-time profiles could best be fitted by a one-compartmental model. The mean percentage of the AUC extrapolated was 1.0±1.1% (range, 0.04-4.2), justifying the use of compartmental methods. After NX 211 administration, peak concentrations were observed at

Table 5.

Pharmacokinetics of Lurtotecan in Plasma as a Function of NX 211 Dose

Dose (mg/m^2)	*n*	C_{max} (ng/mL)	AUC (mg.h/L)	CL ($L/h/m^2$)	V_d (L/m^2)	$T_{1/2}$ (h)
0.4	3	180±25.6	1.21±1.51	0.85±0.64	2.56±0.51	4.29±4.86
		(165-210)	(0.30-2.95)	(0.14-1.36)	(1.97-2.88)	(1.30-9.91)
0.8	6	369±159	1.70±1.92	1.14±1.01	3.36±1.81	3.24±2.69
		(163-629)	(0.28-5.33)	(0.15-2.93)	(1.77-6.29)	(1.51-8.48)
1.6	3	448±131	1.42±0.87	1.46±0.86	5.79±2.26	3.26±0.67
		(306-564)	(0.67-2.37)	(0.67-2.38)	(3.94-8.31)	(2.65-3.99)
3.2	6	1717±441	13.2±10.5	0.41±0.38	2.28±0.85	5.46±3.24
		(1254-2316)	(2.77-33.4)	(0.10-1.15)	(1.45-3.63)	(2.42-11.2)
3.8	6	1517±621	12.0±12.1	0.83±0.71	3.95±2.32	6.81±3.43
		(757-2073)	(2.21-28.0)	(0.14-1.72)	(1.84-7.01)	(2.54-10.8)
4.3	5	2359±857	31.2±16.9	0.51±0.88	6.23±10.4	13.0±7.45
		(886-3130)	(2.08-44.8)	(0.10-2.07)	(1.42-24.8)	(8.58-26.3)
Overall mean	29	-	-	**0.82±0.78**	**3.92±4.43**	**6.24±5.16**

NOTE. Data are expressed as mean ± SD, with observed ranges in parenthesis.

Abbreviations: *n*, number of patients studied; C_{max}, peak concentration; AUC, area under the plasma concentration-time curve; CL, total plasma clearance; V_d, volume of distribution; $T_{1/2}$, terminal disposition half-life.

the end of the 30-minute IV infusion, with an immediate decline following cessation of the infusion. The mean estimated pharmacokinetic parameters for lurtotecan in plasma as calculated by this model are listed in Table 5. Substantial interpatient variability in kinetic parameters was apparent, with a >2-fold variation in peak plasma concentrations and AUC values, although mean values were strongly correlated to dose (C_{max}, Spearman's rho=.94 at *P*=.002; AUC, rho=.89 at *P*=.023). Peak plasma concentrations were also significantly correlated to corresponding AUC values (adjusted R^2=.87 at *P*=.004). A similar degree in variability between patients was evident in total body clearance (CV=95.4%), thereby influencing the actual systemic exposure to lurtotecan during NX 211 treatment. There were no significant differences in (dose-normalized) pharmacokinetic parameters between the various NX 211 dose levels, including the mean total body clearance (*P*=.42; Kruskal-Wallis statistic, corrected for ties). Over the total dose range, the peak concentration and AUC values increased from 180±25.6 (mean±SD) to 236±857 ng/mL and from 1.21±1.51 to 31.2±16.9 mg.h/L, respectively, consistent with a linear and dose-independent kinetic behavior of the drug.

Table 6.
Pharmacokinetics of Lurtotecan in Whole Blood

Parameter		*n*	Mean ± SD	Range
Lurtotecan				
	CL (L/h/m^2)	27	1.15±0.958	0.173-2.93
	V_d (L/m^2)	27	5.74±3.68	2.07-15.0
	$T_{1/2}$ (h)	27	7.00±4.19	1.76-17.3
	B/P ratio	27	0.647±0.134	0.403-1.10

NOTE. Data were obtained from patients treated with NX 211 at dose levels ranging from 0.4 to 4.3 mg/m^2.

Abbreviations: *n*, number of patients studied; CL, total blood clearance; V_d, volume of distribution; $T_{1/2}$, terminal disposition half-life; B/P ratio, AUC ratio of lurtotecan in whole blood and plasma.

Disappearance of lurtotecan from the central plasma compartment was characterized by elimination in an apparent mono-exponential fashion, with a very slow total body clearance of 0.82±0.78 L/h/m2, typical of liposomal-formulated agents [19]. The estimated terminal disposition half-life was relatively consistent in all patients, exhibiting a mean value of 6.24±5.16 hours (CV=82.7%), and was not dependent on the NX 211 dose (P=.42). As a

result of the slow clearance, extended persistence of lurtotecan was apparent, with detectable levels of the compound even at 4 days after initial treatment in most patients. The volume of distribution of lurtotecan was extremely low and averaged 3.92±4.43 L/m2, suggesting distribution takes place mainly within the central compartment, with slow distribution to extravascular tissues. The concentration-time course of lurtotecan in whole blood followed the same general pattern as in plasma, although concentrations were always well below corresponding plasma levels (Fig 2). The AUC ratio of lurtotecan in whole blood and plasma was 0.647±0.134 (n=27), and is indicative for liposomal encapsulation of lurtotecan in the plasma compartment, with no appreciable drug accumulation into erythrocytes (Table 6).

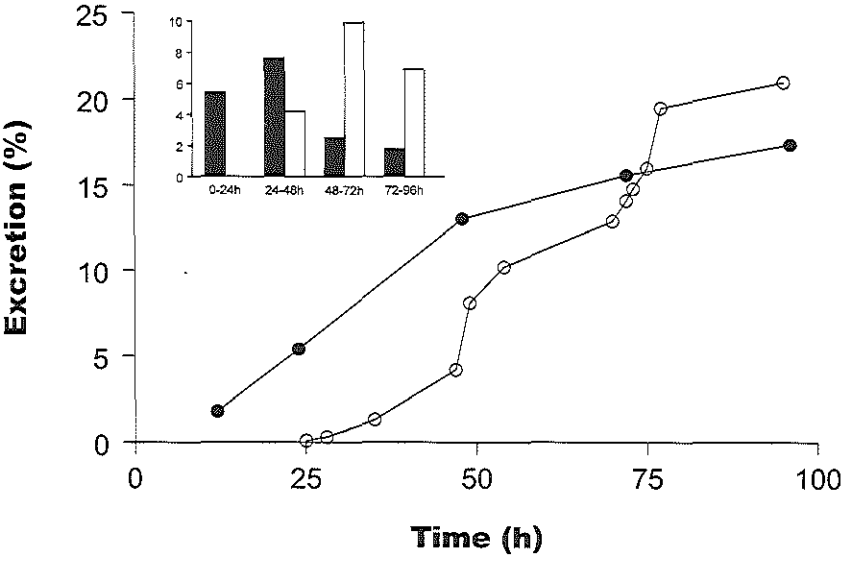


Figure 3. *A) Representative cumulative excretion of unchanged lurtotecan in urine (closed circles) and feces (open circles) in a single patient after administration of NX 211 at a dose level of 3.8 mg/m^2.*
B) Representative excretion of unchanged lurtotecan in urine (closed bars) and feces (open bars) in a single patient after administration of NX 211 at a dose level of 3.8 mg/m^2.

Urinary and Fecal Recovery

Complete data on urinary excretion were available from 23 of 29 patients, whereas data on fecal excretion could be collected in 9 patients. The time course of the cumulative urinary and fecal elimination of lurtotecan for a representative patient is depicted in Fig 3. The urinary excretion pattern was virtually identical in all patients, with approximately 10% (range, 4.90-18.7) of the dose excreted in the first 72 hours and only little after this time. The time course of the fecal excretion was more variable, with most of the compound excreted from 48 to 72 hours after the NX 211 infusion. Although samples were collected for only 96 hours following drug administration, the shown data render it unlikely that the cumulative excretion in either urine or feces will change significantly after this time period.

The total cumulative urinary excretion of lurtotecan accounted for 10.1±4.05% (mean±SD) of the dose in all patients, and was independent of the NX 211-dose. Surprisingly, fecal excretion represented only 10.2±9.28% (range, 3.28-31.4) of the dose, leading to a total recovery of approximately 20% (range, 8.50-46.5) of the dose. This suggests that lurtotecan is extensively metabolized into unknown degradation products. Analysis of the HPLC chromatograms from fecal extracts revealed 2 major additional peaks in all 9 patients (absent in fecal extracts obtained prior to NX 211 treatment), that might represent lurtotecan metabolites (data not shown).

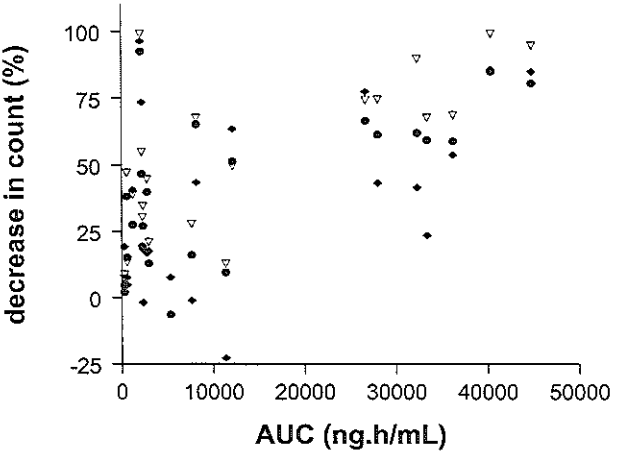


Figure 4. *Relationships between lurtotecan AUC in plasma values of individual patients and the percent decrease in WBC (●), ANC (▽) and platelet count (◆) at nadir relative to pretreatment values. Data were obtained from 29 patients treated with a 30-minute IV infusion of NX 211 at dose levels ranging from 0.4 to 4.3 mg/m^2.*

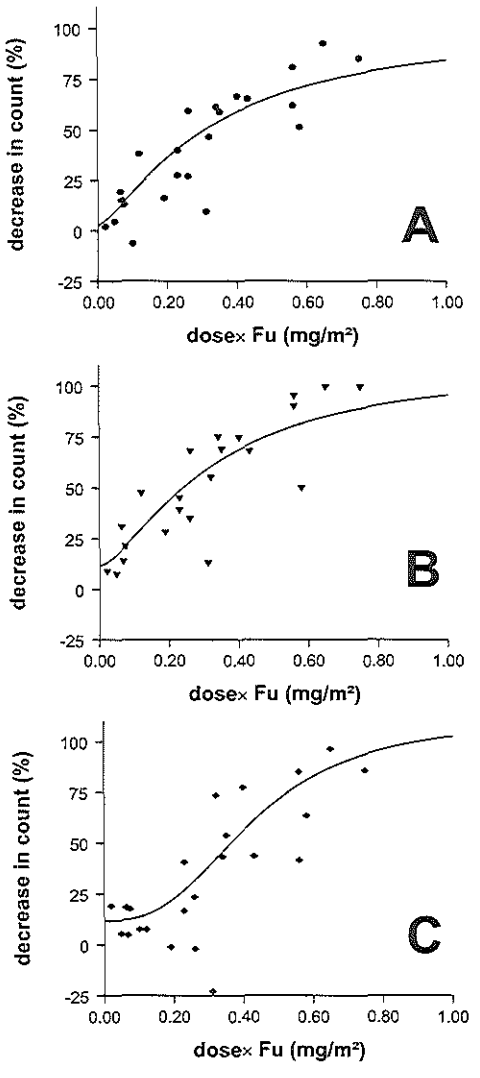


Figure 5. *Relationships between lurtotecan dose×Fu values of individual patients and the percent decrease in (A) WBC, (B) ANC and (C) platelet count at nadir relative to pretreatment values. Data were obtained from 23 patients treated with a 30-minute IV infusion of NX 211 at dose levels ranging from 0.4 to 4.3 mg/m^2. The lines represent the fit of the data to a sigmoid maximum-effect model.*

Hematological Pharmacodynamics

Pharmacokinetic-pharmacodynamic relationships between systemic exposure measures of lurtotecan and hematologic toxicity, including WBC, ANC and platelets, were evaluated using sigmoid maximum-effects models, by plotting kinetic data against the percent decrease in blood cell count at nadir relative to the pretreatment value. The AUC values of lurtotecan in plasma or whole blood were not significantly related to hematologic toxicity and/or misspecifications were noted in the models tested (P>.05) (Fig. 4). Based on the known available pharmacokinetic characteristics of lurtotecan, we hypothesized that unbound (i.e., non-liposomal) lurtotecan concentrations would be more closely related to side effects than to total drug levels, and that an estimate or surrogate measure of exposure to unbound lurtotecan could be obtained from the dose excreted in urine (dose×Fu, expressed in mg/m^2). Indeed, we found that this parameter was significantly correlated to the percent decrease in WBC [(dose×Fu)$_{50}$=0.33±0.083 mg/m^2 (CV=25.0%); γ=1.35±0.350; P<.00001; R^2=.86)], ANC [(dose×Fu)$_{50}$=0.33±0.087 mg/m^2 (CV=26.5%); γ=1.49±0.422; P<.00001; R^2=.85)] and platelets [(dose×Fu)$_{50}$=0.43±0.069 mg/m^2 (CV=16.0%); γ=2.75±1.02; P<.00001; R^2=.79)], and, overall, patients with a higher values of dose×Fu experienced greater hematologic toxicity (Fig. 5). Since the dose×Fu values obtained over the 96-hour time period were closely related to those obtained using the 0-12 hour (R=.77) or 0-24 hour urine-collection interval (R=.93), we also evaluated whether hematologic toxicity could be predicted from excretion data obtained early after NX 211 administration. Unfortunately, these relationships, although still significant (P≤.012), were substantially weaker (R^2=.51 to .80; CV=44.2 to 74.6%), thereby limiting the general applicability of using urine data to predict NX 211-mediated side effects in future clinical trials.

Discussion

This study represents the results of a Phase I trial of NX 211 in patients with solid tumors. Overall, this study demonstrates that NX 211 given every 3 weeks is well tolerated and that no unexpected toxicity was observed. The DLT of NX 211 given as a single drug administration as a 30-minute IV infusion repeated every 3 weeks, consisted of a combination of neutropenia and thrombocytopenia. For hematologic toxicity a remarkable contrast was noted between the 3.8 mg/m^2 and 4.3 mg/m^2 dose levels. Overall, the non-hematologic toxicity was relatively mild, and consisted mainly of nausea, vomiting and fatigue. These findings on toxicity appear to be similar to the results of other schedules investigated with NX 211 [22], and also largely resemble side effects of other topoisomerase I inhibitors [3].

The pharmacokinetic model currently presented accurately describes the plasma concentration versus time profile of lurtotecan after NX 211 administration, and emphasizes the need to apply appropriate kinetic models with sufficient sampling time points, in this case up to 96 hours, coupled with sensitive analytical procedures for the accurate estimation of

kinetic parameters. In general for topoisomerase I inhibitors prolonged exposure has been associated with an increase in cytotoxicity [3]. Of particular note, the phase I data for free (i.e., non-liposomal) lurtotecan suggest that antitumor activity may be enhanced with prolonged infusion regimens [9,10]. Therefore, the use of a liposomal formulation of lurtotecan may improve efficacy at optimally-defined doses by increasing exposure to the drug.

In our study, we have shown that lurtotecan after NX 211 dosing delineates a linear and dose-independent pharmacokinetic behavior over the dose range studied (0.4-4.3 mg/m^2), in agreement with other tested schedules of NX 211 administration [22]. The disappearance of lurtotecan was characterized by a mono-exponential decline with a terminal disposition half-life in plasma of approximately 6 hour. This is in contrast to the multiphasic elimination from plasma reported for non-liposomal lurtotecan, which displays a terminal half-life estimated as 9.6±4.8 hour in a cohort of 14 patients [6]. The basis for the longer elimination half-life of non-liposomal lurtotecan is most likely due to the slow elimination of the larger fraction of drug initially distributed to tissue. With NX 211, a prolonged association of lurtotecan within circulating intact liposomes in the plasma compartment would be assumed to release free drug over a period of time, quite possibly resulting in the same terminal half-life as lurtotecan, but with concentrations below the lower limit of quantitation of our HPLC assay [21]. The prolonged association of lurtotecan with liposomes is thus likely to mask the true disposition half-life of the free drug, as has been observed previously with other agents, including liposomal daunorubicin (DaunoXome) [23]. The total lurtotecan plasma clearance from NX 211, on average 0.82 L/h/m^2, is approximately 25-fold slower than the clearance of the free drug, which was established at 21.0±9.6 L/h/m^2 [6]. The observed steady state volume of distribution of 3.92±4.43 L/m^2 and the blood to plasma AUC ratio of 0.647±0.134 are indicative for prolonged encapsulation of lurtotecan in the liposomes, which are presumed to be localized in the plasma compartment. In this regard, the clinical pharmacokinetic behavior of NX 211 is similar to that observed in previous clinical trials with other liposome-encapsulated anticancer agents, including anthracyclines (e.g., daunorubicin and doxorubicin) and vinca alkaloids (e.g. vincristine) [23-25].

The observed variability in the pharmacokinetic behavior of lurtotecan after the administration of NX 211 is slightly higher than that reported for the free drug, with an interpatient variability in the plasma clearance of 95.4% for NX 211, versus 46% for free lurtotecan, while these values for the volume of distribution were 113% and 52%, respectively [6]. Interestingly, after correction for the body-surface area of individual patients, the interpatient variability in clearance remained in a similar order of magnitude (95.4 versus 98.8%), suggesting that body-surface area is not a significant predictor of lurtotecan clearance and that flat-dosing regimens might be applied in future studies without compromising overall safety profiles.

The cumulative urinary excretion of unchanged lurtotecan of approximately 10% is very well consistent with data of previous studies in which non-liposomal lurtotecan was administered, indicating that renal clearance plays a minor role in drug elimination [5,6]. The mean renal clearance of lurtotecan, i.e., the product of the dose-fraction excreted in urine and the total body clearance, was estimated to be 0.074±0.075 L/h/m^2 (range, 0.008-0.313). This value is much lower than the glomerular filtration rate in humans, presumably due to the association of the drug with the liposomes and binding of free drug to plasma proteins, and suggests that lurtotecan is neither reabsorpted nor actively secreted into the tubular lumen to any great extent. It also indicates that as much as 89.9% (range, 81.3-95.1) of the overall clearance can be attributed to non-renal processes, including hepatobiliary secretion of lurtotecan. Indeed, part of the non-renal elimination was accounted for by fecal excretion of unchanged lurtotecan. However, since the total amount of lurtotecan in feces amounted to only 10% of the administered dose, leading to a total recovery of approximately 20%, lurtotecan is probably extensively metabolized.

An important question that remains unanswered is whether monitoring of extra-liposomal lurtotecan in the systemic circulation would aid in deriving exposure measures more closely linked to NX 211-induced side effects. The rationale for measurement of free-drug concentrations is founded on the basic pharmacologic tenet that agents associated with drug-carrier systems or other macromolecules such as serum proteins are unable to cross cell membranes and interact with extravascular (active) sites. The current finding that various commonly applied exposure measures (e.g. AUCs in plasma and whole blood) were not predictive for hematologic toxicity further substantiates this concept. We have previously shown that the inherent instability of the current NX 211 formulation in aqueous solutions under laboratory light renders it extremely difficult to develop analytical methodologies that allow separation of free and liposomal lurtotecan [21]. Since knowledge of the extent of binding of lurtotecan within the circulation was considered of crucial importance for a proper understanding of the clinical pharmacologic behavior of this drug, we set out to define a surrogate measure that could be linked to the (dose-limiting) toxicity of NX 211. We hypothesized that a dose-corrected urinary-excretion fraction of unchanged drug within a certain time span would reflect systemic exposure to non-liposomal lurtotecan in each individual patient. The calculated parameter (i.e., dose×Fu) was indeed clearly related to pharmacodynamic outcome of NX 211 treatment in terms of hematologic toxicity, and a sigmoid maximum-effect model was found most appropriate to fit the kinetic data to the observed myelosuppression. Considering this pharmacokinetic-pharmacodynamic relation, a target dose×Fu could be defined prospectively according to the grade of toxicity that is considered to be acceptable, and applied in future studies to determine optimal dosing with NX 211 treatment in this schedule. Currently, the suitability of this relationship will be further explored in other dosing schedules with NX 211 [22].

In conclusion, in this phase I study with IV administration of liposomal lurtotecan (NX 211) given once every 3 weeks, the DLT is a combination of neutropenia and thrombocytopenia. The recommended dose for phase II studies with NX 211 in this regimen is 3.8 mg/m^2. Objective responses were not observed, but 9 patients had stable disease while one of these patients experienced a tumor regression of 49%. We have found a pharmacologic-pharmacodynamic relationship for this liposomal encapsulated drug calculated as the dose corrected urinary excretion in relation to hematologic toxicity. Moreover, we have shown that administration of this formulation significantly reduces the plasma clearance of lurtotecan, which in turn might prove beneficial for pharmacodynamic outcome.

References

1. Luzzio MJ, Besterman JM, Emerson DL, et al. Synthesis and antitumor activity of novel water soluble derivatives of camptothecin as specific inhibitors of topoisomerase I. J Med Chem. 1995; 38:395-401.
2. Gerrits CJ, de Jonge MJ, Schellens JH, et al. Topoisomerase I inhibitors: the relevance of prolonged exposure for present clinical development. Br J Cancer. 1997;76:952-962.
3. Kehrer DFS, Soepenberg O, Loos WJ, et al. Modulation of camptothecin analogues in the treatment of cancer: A review. Anticancer Drugs. 2001;12:89-105.
4. Emerson DL, Besterman JM, Brown HR, et al. In vivo antitumor activity of two new seven-substituted water-soluble camptothecin analogues. Cancer Res. 1995;55:603-609.
5. Gerrits CJ, Creemers GJ, Schellens JH, et al. Phase I and pharmacological study of the new topoisomerase I inhibitor GI147211, using a daily x 5 intravenous administration. Br J Cancer. 1996;73:744-750.
6. Eckhardt SG, Baker SD, Eckardt JR, et al. Phase I and pharmacokinetic study of GI147211, a water-soluble camptothecin analogue, administered for five consecutive days every three weeks. Clin Cancer Res. 1998;4:595-604.
7. Gamucci T, Paridaens R, Heinrich B, et al. Activity and toxicity of GI147211 in breast, colorectal and non-small- cell lung cancer patients: an EORTC-ECSG phase II clinical study Ann Oncol. 2000;11:793-797.
8. Sessa C, Wanders J, Roelvink M, et al. Second-line treatment of small-cell lung cancer with the camptothecin- derivative GI147211: a study of the EORTC Early Clinical Studies Group (ECSG). Ann Oncol. 2000;11:207-210.
9. Paz-Ares L, Kunka R, DeMaria D, et al. A phase I clinical and pharmacokinetic study of the new topoisomerase inhibitor GI147211 given as a 72-h continuous infusion. Br J Cancer. 1998;78:1329-1336.
10. Stevenson JP, DeMaria D, Sludden J, et al. Phase I/pharmacokinetic study of the topoisomerase I inhibitor GG211 administered as a 21-day continuous infusion. Ann Oncol. 1999;10:339-344.
11. Hertzberg RP, Caranfa MJ, Holden KG, et al. Modification of the hydroxy lactone ring of camptothecin: inhibition of mammalian topoisomerase I and biological activity. J Med Chem. 1989;32:715-720.
12. Burke TG, Gao X. Stabilization of topotecan in low pH liposomes composed of distearoylphosphatidylcholine. J Pharm Sci. 1994;83:967-969.
13. Subramanian D, Muller MT. Liposomal encapsulation increases the activity of the topoisomerase I inhibitor topotecan. Oncol Res. 1995;7:461-469.

14. Lundberg BB. Biologically active camptothecin derivatives for incorporation into liposome bilayers and lipid emulsions. Anticancer Drug Des. 1998;13:453-461.
15. Sadzuka Y, Hirotsu S, Hirota S. Effect of liposomalization on the antitumor activity, side-effects and tissue distribution of CPT-11. Cancer Lett. 1998;127:99-106.
16. Sadzuka Y, Hirotsu S, Hirota S. Effective irinotecan (CPT-11)-containing liposomes: intraliposomal conversion to the active metabolite SN-38. Jpn J Cancer Res. 1999;226-232.
17. Colbern GT, Dykes DJ, Engbers C, et al. Encapsulation of the topoisomerase I inhibitor GL147211C in pegylated (STEALTH) liposomes: pharmacokinetics and antitumor activity in HT29 colon tumor xenografts. Clin Cancer Res 1998;4:3077-3082.
18. Tardi P, Choice E, Masin D, et al. Liposomal encapsulation of topotecan enhances anticancer efficacy in murine and human xenograft models. Cancer Res. 2000;60:3389-3393.
19. Mayer LD, Cullis PR, Bally MB. Designing therapeutically optimized liposomal anti-cancer delivery systems: lessons from conventional liposomes, in Lassic D, Papahadjopoulos D (eds): Medical Applications of Liposomes, Amsterdam, Elsevier Press, 1998, pp 231-256.
20. Emerson DL, Bendele R, Brown E, et al. Antitumor efficacy, pharmacokinetics, and biodistribution of NX 211: a low-clearance liposomal formulation of lurtotecan. Clin Cancer Res. 2000;6:2903-2912.
21. Loos WJ, Kehrer DFS, Brouwer E, et al. Liposomal lurtotecan (NX211): determination of total drug levels in human plasma and urine by reversed-phase high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 2000;738:155-163.
22. Rothenberg ML, Gelmon KA, Eisenhauer E, et al. Phase I evaluation of liposomal topoisomerase I inhibitor, NX 211, given on days 1,2,3 or days 1,8 every 3 weeks to patients (PTS) with advanced solid tumors. Proc Am Soc Clin Oncol 2000;19:769 (abstr).
23. Pea F, Russo D, Michieli M, et al. Liposomal daunorubucin plasmatic and renal disposition in patients with acute leukemia. Cancer Chemother Pharmacol. 2000;46:279-286.
24. Rahman A, Treat J, Roh JK, et al. A phase I clinical trial and pharmacokinetic evaluation of liposome-encapsulated doxorubicin. J Clin Oncol. 1990;8:1093-1100.
25. Gelmon KA, Tolcher A, Diab AR, et al. Phase I study of liposomal vincristine. J Clin Oncol. 1999;17:697-705.

Chapter 4

Structural Identification and Biological Activity of 7-Methyl-10,11-Ethylenedioxy-20(S)-Camptothecin, a Photodegradant of Lurtotecan

W.J. Loos, J. Verweij, D.F.S. Kehrer, P. de Bruijn, F.M.H. de Groot, M. Hamilton, K. Nooter, G. Stoter, A. Sparreboom

Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam, the Netherlands; Department of Organic Chemistry, NSR-Center for Molecular Structure, Design and Synthesis, University of Nijmegen, the Netherlands; Gilead Sciences Incorporation, Boulder, CO, USA.

Submitted

Abstract

An additional chromatographic peak was observed in plasma samples of patients receiving NX 211, a liposomal formulation of the topoisomerase I inhibitor lurtotecan. We have isolated and purified this product by sequential solid-phase extractions, and we report its structure and cytotoxicity relative to lurtotecan and related agents. NMR data indicate that cleavage of the piperazino moiety occurred at the N-C bond of the B-ring, yielding 7-methyl-10,11-ethylenedioxy-20(*S*)-camptothecin (MEC). Tests of the growth inhibition potential of MEC in 7 human tumor cell lines showed that the compound was approximately 2 to 18-fold more cytotoxic than lurtotecan, topotecan and SN-38. Subsequently, we found that MEC was the product of rapid photolysis of lurtotecan, with the rate of degradation inversely proportional to NX 211 concentrations, and greatly depend on light intensity. Furthermore, MEC concentrations were found to significantly increase in plasma samples exposed to laboratory light, but not in blood. MEC was not produced from NX 211 in the presence of human liver microsomes, suggesting that is not a product of CYP P450 metabolism. Using a validated analytical method, trace levels of MEC were quantitated in blood samples of 2 patients. These observations confirm that the precautions for protection form light currently specified for preparation and administration of NX 211 dose solution are critical. Procedures to minimize formation of MEC, by the use of amber vials for NX 211 and by preparation of dilutions immediately before clinical use in a fashion totally protected from light, are now being routinely implemented.

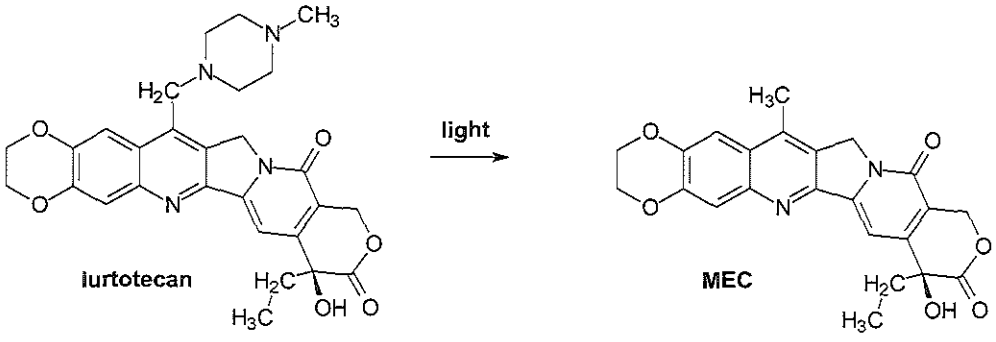


Figure 1. *Chemical structures of lurtotecan and its photodegradant MEC.*

Introduction

The currently known analogues of camptothecin, a cytotoxic plant alkaloid from *Camptotheca acuminata*, share a basic pentacyclic structure with a chiral center located at C20 in the terminal E-ring (Fig. 1). Extensive studies on the synthesis of these derivatives and the development of structure-activity relationships have been carried out over the last several years, and some important general relationships have emerged [1]. One of the principal chemical features of this class of agents is the presence of a lactone functionality in the E-ring, which is not only essential for antitumor activity, but it also confers a degree of instability to these agents in aqueous solutions [2]. The camptothecins can undergo a pH-dependent reversible interconversion between this lactone form and a ring-opened carboxylate (or hydroxy acid) form, of which only the former exerts the characteristic topoisomerase I-inhibitory activity. In the search for more potent camptothecin analogues, the synthetic preparation of hexacyclic derivatives has been described, and two representative agents of this class, lurtotecan and exatecan mesylate (DX-8951f) are currently undergoing clinical evaluation [3,4]. The former is a water-soluble derivative with a dioxalane moiety between C10 and C11 [5], and has been evaluated clinically in various phase I and phase II trials using a 30-min i.v. infusion given daily for 5 days or as a 72-h continuous i.v. infusion [6-8]. Alternative strategies of lurtotecan administration are currently being developed, including a new liposomal formulation (NX 211) to further stabilize the lactone moiety and induce sustained drug levels that may be required to maximize antitumor activity [9,10]. We previously reported the presence of an unidentified compound in the plasma of patients treated with NX 211 as well as in control samples spiked with the liposomal product [11]. Since the native fluorescence intensity of the parent drug (i.e., lurtotecan) was previously shown to be sensitive to UV-light exposure (254 nm) and the fact that the peak height of the newly generated compound was correlated with the lurtotecan concentration, we suspected photodegradation of lurtotecan to produce an additional species. In the current study, we have isolated this unknown compound by using HPLC coupled to fluorometric detection followed by NMR analysis of the purified fraction. Next, the properties of this compound in terms of cell growth inhibition relative to related analogues were examined in addition to the kinetics of lurtotecan photolysis in various aqueous and biological solutions.

Materials and Methods

Chemicals

Lurtotecan and NX 211 were supplied by Gilead Sciences (San Dimas, CA). Topotecan was a gift from Smith-Kline Beecham Pharmaceuticals (King of Prussia, PA), while SN-38 was supplied by Aventis (Alfortville, France). RPMI cell culture medium, penicillin, streptomycin and *l*-glutamine were purchased from Life Technologies (Gaithersburg, MD) and fetal

calf serum was delivered by Hyclone (Logan, UT). ACN and MeOH, both HPLC-grade, were obtained from Biosolve (Valkenswaard, The Netherlands) and DMSO from Rathburn (Walkerburn, UK). Baker (Deventer, The Netherlands) delivered TCA, HAC (99-100%), PCA (70%), and HCl (36-38%) and ammonium acetate was from Roth (Karlsruhe, Germany). Sulforhodamine B and NADPH were obtained from Sigma (St. Louis, MO). PBS was obtained from Oxoid and supplied by Boom (Meppel, Netherlands), and consisted of tablets containing sodium chloride (8.0 g/l), potassium chloride (0.2 g/l), disodium hydrogenphospate (1.15 g/l) and potassium dihydrogenphosphate (0.2 g/l), each dissolved in 100 mL purified water. All water used in the study was filtered and deionized with a Milli-Q-UF system (Millipore, Milford, MA).

Isolation and Purification

MEC was prepared by exposure of 17 mg of the lactone form of LRT in a total volume of 40 mL DMSO-0.1 M ammonium acetate pH=5.5 (1:4, v/v) to UV light of 254 nm in a Beam-Boost photochemical reactor unit (ICT-ASS-Chem, Bad Homburg, Germany) for 5 min. After irradiation, the sample was acidified with 400 µl PCA and applied onto 4 C18 solid-phase extraction cartridges (Applied Separations, Allentown, PA). Subsequently, the cartridges were washed 3 times with 1 mL 25 mM ammonium acetate pH=3.0, followed by 3 times with 1-mL volumes of a mixture of 25 mM ammonium acetate pH=3.0-MeOH (6:4, v/v). The columns were eluted with three 1-mL volumes of 25 mM ammonium acetate pH=3.0-MeOH (1:9, v/v), followed by three 1-mL volumes of MeOH. The eluate was dried at 60°C under nitrogen until approximately 1 mL was left over, which resulted in a precipitate in the sample. In order to resuspend the precipitate, the sample was diluted with 10% PCA-ACN-PBS (2/1/6, v/v/v) to 12 mL, and 200-µL portions of this mixture were injected onto the HPLC-system as described previously [11] with the flow rate set at 3.75 mL. The peaks with a retention time of 17 min were collected, in a total volume of approximately 1 L, which was acidified with 10 ml PCA and filled with water up to 2 L. Further purification was achieved on C18 solid-phase extraction cartridges, which were washed 3 times with 1 mL water, followed by elution with 5 volumes of 1 mL MeOH. The eluate fractions were collected and lyophilized using a FDC206 Freeze drying Chamber (Savant, Farmingdale, NY) to obtain the pure compound for chemical and biological characterization.

Structural Analysis

The isolated powder was characterized by 1H-NMR spectrum, measured on a Bruker AM-300 (300 MHz, FT) spectrometer. Chemical shift values are reported as δ-values in parts per million using DMSO-D6 (2.5 ppm) as a standard.

Cytotoxicity Assays

The human colon carcinoma WiDr, the ovarian adeno-carcinoma IGROV-1, the M-19 melanoma, the H226 lung cancer, the renal cancer cell A498, and the MCF7 and EVSAT breast cancer cell lines were grown and maintained in RPMI medium. Cells were kept in continuous logarithmic growth at 37°C in a humidified atmosphere in 5% CO_2 in media supplemented with 10% (w/v) of heat-inactivated fetal calf serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin and 2 mM *l*-glutamine. Exponentially grown cells were trypsinized and plated (200 µL with 2000 cells/well) in 96-well culture plates (Costar Corporation, Cambridge, MA), 48 hours before drug exposure. The stock solution of MEC was diluted in a mixture of DMSO/HCl (98:2, v/v), resulting in a concentration of 18 µM, while lurtotecan, topotecan and SN-38 were dissolved separately in DMSO/HCl (98:2, v/v) to obtain concentrations of 180 µM. Prior to addition to the cells, the solutions were diluted 20-fold in culture medium, resulting in concentrations of 0.90 µM of MEC and 9.0 µM of lurtotecan, topotecan and SN-38. A volume of 100 µL of these solutions was added to the cells, followed by 3-fold serial dilutions in the 96-well plate. Subsequently, the plates were incubated for a period of 5 days. For comparative purposes, MEC, lurtotecan, topotecan and SN-38 were evaluated in parallel in all experiments. After fixation with 10% (w/v) aqueous TCA, inhibition of cell proliferation was assessed using sulforhodamine B staining as described [12], with minor modifications [13]. Each compound was tested in quadruplicate in 3 independent experiments. Cell survival was plotted relative to controls incubated in medium in the absence of the drugs. IC_{50} values were calculated after fitting the individual curves to a Hill function using the software package Siphar version 4.0 (SIMED, Créteil, France).

Analytical Methods

Total lurtotecan concentrations in human whole blood were determined using a validated HPLC method (to be published elsewhere). In brief, aliquots of 50 µL heparinized whole blood were pretreated with 500 µL of 5% (w/v) aqueous PCA-ACN (5:1, v/v), followed by vigorous vortex-mixing for 30 min. Subsequently, the samples were centrifuged for 5 min at 23,000 x *g*. The HPLC system was identical to that described previously for the determination of lurtotecan concentrations in urine [11], with the flow-rate set at 1.25 mL/min. For determination of MEC in whole blood samples the HPLC system consisted of a constaMetric 3200 solvent delivery system (LDC Analytical, Riviera Beach, FL), a Waters 717plus autosampler (Milford, MA) from which the sample-tray was protected from light, a Inertsil-ODS 80A analytical column (150 x 4.6 mm internal diameter; 5 µm particle size; Alltech Applied Sciences, Breda, The Netherlands) maintained at 60°C by a model SpH99 column oven (Spark Holland, Meppel, The Netherlands), and a Jasco FP-920 fluorescence detector (Jasco, Maarssen, The Netherlands) operating at excitation and emission wavelengths of 378 and 420 nm (40 nm band width), respectively. The mobile phases consisted of 1 M

ammonium acetate-water-ACN (1:6:3, v/v/v), with the pH adjusted to 5.5 and the flow rate was set at 0.50 mL/min.

MEC was extracted from 50-µL aliquots of heparinized whole blood using 250 µL of a mixture of MeOH/ACN (1:1, v/v) in 1.5-mL polypropylene tubes (Eppendorf, Hamburg, Germany). The samples were vigorously vortex-mixed for 5 min on a multi-tube vortex mixer, followed by centrifugation for 5 min at 23,000 x g at ambient temperature. A volume of 150 µL of the clear supernatant was transferred to a new 1.5-mL polypropylene tube, followed by addition of 250 µL of 25 mM ammonium acetate pH=3.0. Subsequently, a volume of 300 µL was transferred to a low volume insert of glass, from which 200 µL were injected into the HPLC system. The calibration curves were constructed in human heparinized whole blood in concentrations of 0.10, 0.25, 1.00, 2.50, 5.0 and 10.0 ng/mL, by serial dilutions in PBS of a MEC working solution containing 10 µg/ml in DMSO. Four pools of QC samples containing MEC were prepared in heparinized whole blood at concentrations of 0.40, 4.00, 8.00 and 80.0 ng/mL MEC, by addition of appropriate volumes of dilutions of MEC in PBS to whole blood. A fifth QC sample containing 800 ng/mL NX 211 was also prepared in heparinized whole blood, to minimize potential differences with clinical samples.

Validation of the assay included a set of calibration samples assayed in duplicate, with LLQ and QC samples in quintuplicate, and was performed on 4 separate occasions. Within-run and between-run precisions were calculated by one-way analysis of variance for each concentration, using the run-day as variable. The extraction recovery for MEC was calculated by comparing peak heights obtained from processed samples containing 2.50 ng/mL MEC in PBS to those obtained in extracted calibration samples.

Stability of NX 211

The increase of MEC in plasma and whole blood containing NX 211 (800 ng/mL) was tested on 3 separate occasions at ambient temperature, by placing 250-µL aliquots of the samples in the dark and in sodium light (lamp: Philips SON-T-Plus 70 W), laboratory (lamp: Philips TLD 36W) and in sunlight. After a 2-h incubation, 50-µL aliquots were stored in duplicate at –80°C until analysis. The plasma and whole blood concentrations of MEC were determined using the method described above and were compared to MEC concentrations in non-incubated samples. The increase of MEC in the extracts of the QC sample containing NX 211 (800 ng/mL) was tested by placing the extracts on 3 separate occasions at ambient temperature in laboratory and sodium light. After 1 and 2-h incubation periods, the extracts were placed in the autosampler and peak heights were compared to those of extracts stored in the dark. The light sensitivity of the clinical formulation of NX 211 was tested by placing serial dilutions of NX 211 in D5W in the dark, and in sodium and laboratory light. After 2 h, 50-µL aliquots were stored in duplicate at -80°C and were analyzed for MEC concentrations using the assay described. The concentrations of MEC in the incubated samples were compared to non-incubated serial dilutions of NX 211 in D5W.

To rule out a potential metabolic conversion of lurtotecan into MEC, 1-mL aliquots of (1 mg/mL) pooled human liver microsomes (Gentest, Woburn, MA) were incubated in the dark in a phosphate buffer of pH=7.4 at 37°C with 2 μM of lurtotecan or NX 211 in the presence of 3 μM NADPH. After a 1-h incubation period, 50 μL aliquots were analyzed for MEC concentrations resulting from metabolic instability, as described for whole blood samples.

Clinical Samples

Whole blood samples for pharmacokinetic analysis were drawn from a vein in the arm opposite to that used for drug infusion from 2 male cancer patients (age, 39 and 64 years) participating in a phase I trial [10], and collected in 4.5-mL glass tubes containing lithium heparin as anticoagulant. Samples were obtained before drug administration and at 0.5 (end of infusion), 1, 1.5, 2.5, 4, 6, 8, 24, 48, 72, and 96 h after start of infusion and were immediately transferred to a polypropylene vial and stored at –80°C. The NX 211 was formulated as a sterile liposomal dispersion of lurtotecan in a buffer composed of 10 mM ammonium chloride and 9% sucrose and was supplied in 50-mL clear glass vials containing 5 mg of the active agent. Each vial contained 80 mg hydrogenated-soy phosphatidylcholine, 19 mg cholesterol, 0.9 g sucrose, 2 mg citric acid, and 5 mg ammonium chloride to a total volume of 10 mL. Dose solutions for administration were prepared in polyvinyl chloride-free infusion syringes under aseptic conditions by dilution of the pharmaceutical preparation with D5W to a volume of 25 mL. The solution was delivered by a 30-min intravenous infusion, protected from light by wrapping the syringe in aluminum foil. Small aliquots of the NX 211 solutions were stored at –80 °C for analysis of MEC and total lurtotecan [11] concentrations. The current clinical protocol was approved by the Rotterdam Cancer Institute Review Board, and both patients signed informed consent before study entry.

Results

Structural Identification

The principal unknown peak observed in plasma samples of patients receiving NX 211 [11] was prepared and isolated (0.6 to 0.7 mg) after irradiation of lurtotecan, and dried down to a pale-yellow powdery residue. The purified compound yielded NMR spectra with the aromatic part very similar to that of LRT (14); [δ=0.87, t, 3H, J = 7.2 Hz, 19-CH_3; δ=1.87, m, 2H, 18-CH_2; δ=2.67, s, 3H, B-ring CH_3; δ=4.43, s, 4H, O-CH_2-CH_2-O; δ=5.22 and δ=5.42, 2×s, 2×2H, CH_2's 5 and 17; δ=7.25, s, 1H, Ar-H; δ=7.54, s, 1H, Ar-H; δ=7.59, s, 1H, Ar-H]. However, the 8 protons of the piperazino group on the B-ring at C-7 at δ=2.40 – 2.65 (m) were absent in the isolated product. The signal at δ=2.67 (s, 3H), characteristic of an *n*-methyl substituent suggested that scission of the piperazino group had occurred at the N-C bond of the B-ring, and thus could be assigned to MEC (Fig. 1). Electrospray ion-trap mass-spectrometric analysis also already indicated the loss of the C-7 side chain of the lurtotecan

molecule [molecular ion at *m/z* 409 (parent – C-7 chain)] (11). The chromatographic purity of the compound on the reversed-phase system was found to be >99% (data not shown).

In Vitro Cytotoxicity

The cytotoxic properties of MEC relative to lurtotecan and several clinically important analogues were assessed by exposure of each test compound to a panel of 7 cell lines. Under the experimental conditions applied, MEC was found to be a highly potent inhibitor of cell proliferation in all cell lines. Among the tested topoisomerase I inhibitors lurtotecan, topotecan and SN-38, MEC was found to be the most potent inhibitor of cell-growth during a 5-days continuous exposure (Table 1). On average MEC was 3.4 ± 0.88 fold more potent than lurtotecan, 3.5 ± 1.1 fold more potent than SN-38 and 13 ± 3.0 fold more potent than topotecan in the 7 tested cell lines.

Table 1.
Comparative in vitro cytotoxicity of MEC and structural analogues.

Cell line	MEC IC-50 (nM)	lurtotecan IC-50 (nM)	F[a]	SN-38 IC-50 (nM)	F	topotecan IC-50 (nM)	F
WIDr	2.4 ± 0.035	7.6 ± 1.8	3.1	8.0 ± 0.87	3.3	27 ± 1.9	11
IGROV-1	1.6 ± 0.023	5.4 ± 0.67	3.3	3.5 ± 0.92	2.2	19 ± 2.9	12
M19	25 ± 2.1	80 ± 65	3.2	117 ± 96	4.6	271 ± 185	11
H226	8.1 ± 0.55	15 ± 1.9	1.8	34 ± 40	4.2	103 ± 51	13
A498	3.3 ± 0.17	13 ± 11	4.0	8.5 ± 1.4	2.6	32 ± 7.7	9.7
MCF-7	1.3 ± 0.022	4.3 ± 1.63	3.5	3.5 ± 0.80	2.8	19 ± 4.3	15
EVSAT	8.1 ± 0.58	38 ± 30	4.7	41 ± 31	5.0	147 ± 46	18

[a]Abbreviation: F, fold less toxic than MEC.

Stability of NX 211

To gain insight into the degradation kinetics of lurtotecan, as a potential source of MEC formation, experiments were conducted under various conditions and light intensities. In plasma as well as in whole blood, MEC was formed out of NX 211 when incubated for 2-h at ambient temperature in sunlight, with, respectively, a 99-fold and 4.5-fold increase in MEC concentration as compared to samples stored at –80°C (Fig. 2). Plasma samples placed under sodium light and laboratory light also showed increased MEC concentrations, while in blood no increase was observed under the same conditions (Fig. 2). An increase in the concentration of MEC also was found after incubation of blood-extracts at ambient temperature under

sodium light and laboratory light (Fig. 2A). Increased concentrations of MEC in the clinical formulation of NX 211 were observed after a 2-hr incubation at ambient temperature under sodium- and laboratory light. After dilution of this clinical formulation in D5W, the light sensitivity of NX 211 was increased (Fig. 2B). In addition, no increase in MEC concentrations were found after a 1-h incubation period of lurtotecan or NX 211 in pooled human liver microsomes, suggesting that the compound was not sensitive to hepatic metabolic degradation.

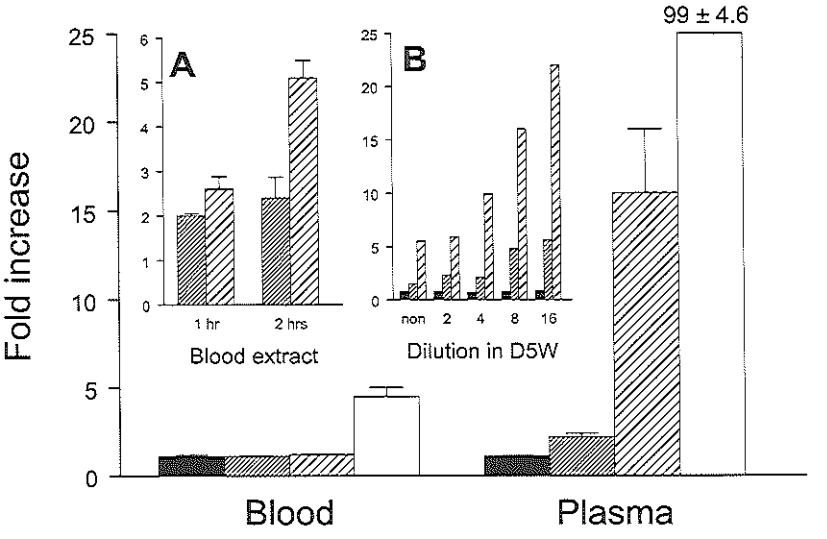


Figure 2. *Stability of lurtotecan in human whole blood and plasma spiked with NX 211 (800 ng/mL), blood extracts (insert A) and aqueous dilutions of NX 211 in D5W (insert B) expressed as fold increase in MEC formation relative to a control sample (nominal). Data are presented as mean values (bar) ± SD (error bar). The various bars refer to the various incubation/light intensity conditions: black, dark; gray, sodium light; hatched, laboratory light; white, sunlight.*

HPLC Analysis

To obtain information on the pharmacological and toxicological relevance of the lurtotecan photolysis, we set out to develop an analytical method to allow quantitative determination of MEC in samples of patient receiving NX 211 infusions. In view of the relative stability of MEC in whole blood samples, we focused our attention on this matrix to address the pharmacokinetic behavior of the compound. The calibration curves of MEC in

human heparinized whole blood were linear over the studied range of 0.10 to 10 ng/mL, with Pearson's regression coefficients ranging from 0.9986 to 0.9995 using weighted (1/[MEC]) linear least-squares regression analysis. The retention time of MEC was 13 min (overall run time, 15 min), and no endogenous interferences were found in drug-free specimens (Fig. 3).

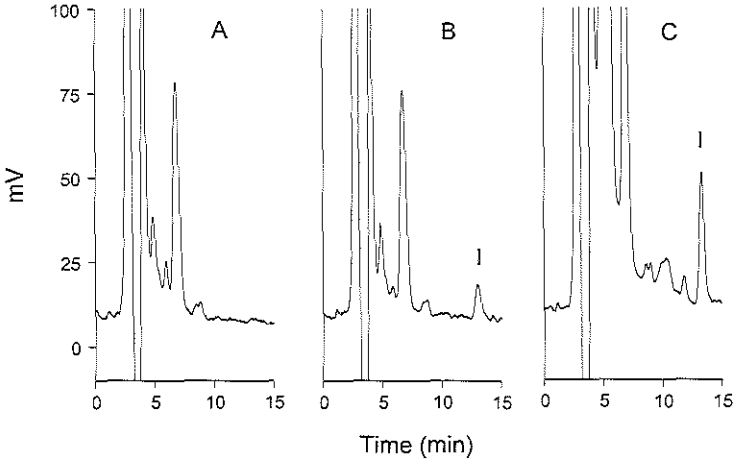


Figure 3. *Reversed-phase HPLC tracings of a blank whole blood extract (panel A), a whole blood sample spiked with MEC (0.25 ng/mL) (panel B), and a whole blood sample spiked with the clinical formulation of NX 211 (800 ng/mL) (panel C). The peak labeled I indicates MEC.*

The LLQ of the method was established at 0.10 ng/mL (~0.24 nM) and the within- and between-run precisions of the LLQ and quality-control samples were ≤7.8%, with the accuracy ranging from 98.4 to 104% (Table 2). The within and between-run precisions of the QC sample containing 800 ng/mL NX 211 were 9.7 and 17%, respectively. All the QC samples were found to be stable during 3 freeze-thaw cycles. The recovery of MEC was constant over the entire range and was 104 ± 11.9 % (n=43). Overall, these validation data of the assay were considered acceptable for its use in clinical studies.

Table 2.
Validation characteristics of QC samples for the HPLC assay of MEC.

Nominal (ng/ml)	Observed (ng/ml)	WRPa (%)	BRP (%)	ACC (%)
0.10b	0.10	7.1	2.7	104
0.40	0.40	7.8	c	101
4.00	4.01	3.4	6.0	100
8.00	8.23	6.0	c	103
80.0	78.7	3.9	7.5	98.4
800 NX 211	0.92	9.7	17	-

a Abbreviations: WRP, within-run precision; BRP, between-run precision; ACC, accuracy.
b Lower limit of quantitation samples.
c No additional variation was observed as a result of performing the assay in different runs.

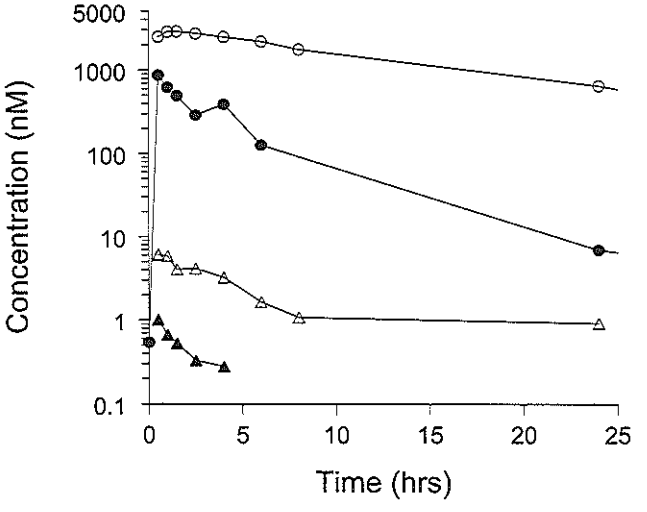


Figure 4. *In vivo pharmacokinetics of total lurtotecan (circles) and MEC (triangles) in whole blood of 2 patients treated with NX 211 [7.3 mg (open symbols) and 7.5 mg (closed symbols)] administered as a 30-min i.v. infusion.*

Patient Studies

The 2 studied patients received 7.5 mg and 7.3 mg NX 211 (14.5 and 14.1 μmol), respectively. The total amount of lurtotecan in the infusion solutions of both patients was established at 6.9 mg (13.2 μmol). The total amount of MEC in the sample taken prior to infusion of patient 1 was 30.1 μg (73.5 nmol), while at the end of the infusion the total amount of MEC was increased to 73.5 μg (180 nmol). Thus, the percentage of NX 211 administered as MEC, on molecular basis, was 0.56% prior to infusion and 1.4% at the end of the infusion. From patient 2 a sample was taken prior to infusion, which contained 75.7 μg MEC (185 nmol), i.e., at least 1.4% of the total amount of drug administered was in the form of MEC. The kinetic profiles of total lurtotecan and MEC obtained from the two patients are shown in Fig. 4.

Discussion

In the present study, we isolated sub-milligram quantities (0.6 to 0.7 mg) of a (highly apolar) circulating compound present in the plasma of patients treated with NX 211, a liposomal preparation of the topoisomerase I inhibitor lurtotecan. Following serial purification steps, the product was eventually obtained as a pale yellow powdery residue, and the use of HPLC in combination with fluorescence detection and NMR enabled us to identify its chemical structure. NMR characteristics of the aromatic part were shown to be very similar to that of lurtotecan, and the isolated product was tentatively identified as MEC. This structural assignment is in accordance with our earlier finding of the loss of the C-7 side chain of the lurtotecan molecule using electrospray ion-trap mass-spectrometric analysis [11].

MEC was found to be a highly potent inhibitor of cell growth in culture following a 5-day continuous exposure in a panel of 7 cell lines, with IC_{50} values on average 3.4, 3.5- and 13-fold lower than those of the parent drug lurtotecan, the irinotecan metabolite SN-38 and topotecan, respectively. These data are consistent with earlier findings of the substantially higher *in vitro* cytotoxic activity of 7-methyl-10,11-methylenedioxy-camptothecin compared to 10,11-methylenedioxy-camptothecin after a 3-day continuous exposure [15]. The former compound is chemically similar to MEC, with a methylenedioxy substitution at the C10,11-positions in the core structure instead of an ethylenedioxy moiety for MEC. The substitution of the methyl moiety at the B-ring thus resulted in enhanced cytotoxicity, which is in agreement with known structure-activity relationships of camptothecin analogues indicating that alkylated substitutions at C7 tend to increase topoisomerase I-inhibitory activity and simultaneously decrease aqueous solubility [16]. Moreover, 7-chloromethyl-10,11-ethylenedioxy-camptothecin was recently found to be a more potent inhibitor of cell growth than lurtotecan and topotecan in several tumor cell lines during a 48-h continuous exposure [14]. In addition, this agent which has an additional Cl-substitution at the methyl-moiety at

the B-ring relative to MEC, was almost insoluble in water, which is also in agreement with the current findings.

MEC is most likely a product of lurtotecan photolysis, as the parent drug was highly sensitive to light-induced degradation, and was not significantly converted to MEC by human liver microsomes. In aqueous solutions, the rate of lurtotecan degradation appeared to be inversely proportional to drug concentration, and was greatly depending on light intensity with no noticeable degradation occurring in the dark and progressively increased instability in the presence of sodium light<laboratory light<sunlight. The lurtotecan degradation kinetics in biological fluids was markedly altered, which is most likely associated with stabilizing effects of matrix components, as has been observed previously for anthracycline antineoplastic agents [17]. As outlined, such effects might be either caused by increased drug stability due to binding to matrix constituents or by quenching of incident light by intense coloring of the sample or the presence of particulate matter (e.g., blood cells). In contrast to the photodegradation of lurtotecan, photolysis of irinotecan (CPT-11), one of the most extensively studied camptothecin analogues, only occurs at the fragile lactone-ring structure (18,19). In view of the close chemical similarity of the lactone moiety between camptothecins, photodegradation of lurtotecan is also likely to occur at this site of the molecule. In general, however, such degradation products are not of major (clinical) interests, since the reactivity of an intact lactone-ring structure is required for the generation of topoisomerase I-mediated DNA cleavage and antitumor activity of all known camptothecin analogues [20].

The question arises if the additional chromatographic peak observed in the plasma samples of patients were the result of photochemical degradation during the blood sample handling and subsequent analytical procedure, or that MEC was co-administered simultaneously with the NX 211 infusate. We found that, *in vitro*, the amount of MEC in plasma samples increased 99-fold, 16-fold and 2.2-fold after a 2-h exposure to sunlight, laboratory light and sodium light, respectively, while after incubation in the dark no increase was observed. In contrast to plasma, MEC concentrations did not increase in whole blood under similar conditions with the exception of sunlight exposure, where incubation resulted in a 4.5-fold increase in MEC concentration. Although it was beyond the scope of this study to assess the chemical basis of this discrepant behavior, the apparent stability of MEC in whole blood samples provided the possibility to study its disposition in patients. The HPLC-method developed for this purpose was shown to be sufficiently accurate and precise to monitor circulating concentrations in patients treated with NX 211. On average, the total amount of MEC in the samples spiked with 800 ng/ml NX 211 (1544 nM LRT) was established at 0.92 ng/ml (2.25 nM). Hence, a small fraction (~0.15%) of the clinical formulation of NX 211 is thus already present in the form of the photochemical degradant MEC.

The lower percentages of MEC present in the blood samples of the 2 studied patients, ranging from 0.061 to 0.24%, compared to values of 0.56 to 1.4% in the NX 211 infusates,

could be indicative for extra-liposomal positioning of MEC. The photostability data presented here also suggests that the infusates are far more sensitive to production of MEC than whole blood under laboratory conditions. Currently, we are conducting a retrospective analysis of whole blood samples in a larger group of cancer patients treated with NX 211 to describe in more detail the pharmacokinetic behavior of MEC.

In conclusion, we have shown that lurtotecan in the liposomal formulation (NX 211) is sensitive to a rapid degradation if exposed to light resulting in formation of a highly cytotoxic compound, that was structurally identified as MEC. The data presented may be of clinical importance as dilutions of NX 211 for use in patients are prepared in aqueous solutions, usually in advance of administrations as 30-min i.v. infusion. Clearly, the presence of this product at high enough concentrations could not only confound in vitro and in vivo cytotoxicity and antitumor activity studies but might distress efforts to elucidate the metabolic fate of lurtotecan and potentially other (C7-substituted) camptothecin derivatives. Procedures to minimize formation of MEC, by the use of amber vials for NX 211 and by preparation of dilutions immediately before clinical use in a fashion totally protected from any light, are currently being routinely implemented.

References

1. Chourpa I, Beljebbar A, Sockalingum GD. Structure-activity relation in camptothecin antitumor drugs: why a detailed molecular characterisation of their lactone and carboxylate forms by Raman and SERS spectroscopies? Biochim Biophys Acta. 1997;1334:349-360.
2. Loos WJ, de Bruijn P, Verweij J, et al. Determination of camptothecin analogs in biological matrices by high- performance liquid chromatography. Anticancer Drugs. 2000;11:315-324.
3. Kehrer DFS, Soepenberg O, Loos WJ, et al Modulation of camptothecin analogues in the treatment of cancer: a review. Anticancer Drugs. 2001;12:89-105.
4. Rowinsky EK, Johnson TR, Geyer CE Jr, et al. DX-8951f, a hexacyclic camptothecin analog, on a daily-times-five schedule: a phase I and pharmacokinetic study in patients with advanced solid malignancies. J Clin Oncol. 2000;18:3151-3163.
5. Emerson DL, Besterman JM, Brown HR, et al. In vivo antitumor activity of two new seven-substituted water-soluble camptothecin analogues. Cancer Res. 1995;55:603-609.
6. Gerrits CJ, Creemers GJ, Schellens JH, et al. Phase I and pharmacological study of the new topoisomerase I inhibitor GI147211, using a daily x 5 intravenous administration. Br J Cancer. 1996;73:744-750.
7. Eckhardt SG, Baker SD, Eckardt JR, et al. Phase I and pharmacokinetic study of GI147211, a water-soluble camptothecin analogue, administered for five consecutive days every three weeks. Clin Cancer Res. 1998;4:595-604.
8. Paz-Ares L, Kunka R, DeMaria D, et al. A phase I clinical and pharmacokinetic study of the new topoisomerase inhibitor GI147211 given as a 72-h continuous infusion. Br J Cancer. 1998;78:1329-1336.
9. Emerson DL, Bendele R, Brown E, et al. Antitumor efficacy, pharmacokinetics, and bio-distribution of NX 211: a low-clearance liposomal formulation of lurtotecan. Clin Cancer Res. 2000;6:2903-2912.
10. Bos A, Kehrer D, Sparreboom A, et al. Phase I study of NX 211 (liposomal lurtotecan) administered as a single dose every 3 weeks. Proc Am Soc Clin Oncol. 2000;19:197a.

11. Loos WJ, Kehrer D, Brouwer E, et al. Liposomal lurtotecan (NX211): determination of total drug levels in human plasma and urine by reversed-phase high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 2000;738:155-163.
12. Skehan P, Storeng R, Scudiero D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst. 1990;82:1107-1112.
13. Sparreboom A, de Jonge MJ, de Bruijn P, et al. Irinotecan (CPT-11) metabolism and disposition in cancer patients. Clin Cancer Res. 1998;4:2747-2754.
14. Luzzio MJ, Besterman JM, Emerson DL, et al. Synthesis and antitumor activity of novel water soluble derivatives of camptothecin as specific inhibitors of topoisomerase I. J Med Chem. 1995;38:395-401.
15. Vladu B, Woynarowski JM, Manikumar G, et al. 7- and 10-substituted camptothecins: dependence of topoisomerase I-DNA cleavable complex formation and stability. Mol Pharmacol. 2000;57:243-251.
16. Tanizawa A, Fujimori A, Fujimori Y, et al. Comparison of topoisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin derivatives presently in clinical trials. J Natl Cancer Inst. 1994;86:836-842.
17. Le Bot MA, Riche C, Guedes Y, et al. Study of doxorubicin photodegradation in plasma, urine and cell culture medium by HPLC. Biomed Chromatogr. 1988;2:242-244.
18. Dodds HM, Craik DJ, et al. Photodegradation of irinotecan (CPT-11) in aqueous solutions: identification of fluorescent products and influence of solution composition. J Pharm Sci. 1997;86:1410-1416.
19. Dodds HM, Robert J, et al. The detection of photodegradation products of irinotecan (CPT-11, Campto, Camptosar), in clinical studies, using high-performance liquid chromatography/atmospheric pressure chemical ionisation/mass spectrometry. J Pharm Biomed Anal. 1998;17:785-792.
20. Chourpa I, Riou JF, Millot JM, et al. Modulation in kinetics of lactone ring hydrolysis of camptothecins upon interaction with topoisomerase I cleavage sites on DNA. Biochemistry. 1998;37:7284-7291.

Chapter 5

Factors Involved in Prolongation of the Terminal Disposition Phase of SN-38: Clinical and Experimental Studies

D.F.S. Kehrer, W. Yamamoto, J. Verweij, M.J.A. de Jonge, P. de Bruijn, A. Sparreboom

Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam, The Netherlands

Clinical Cancer Research, 6: 3451-3458, 2000

Abstract

The active metabolite of irinotecan (CPT-11), 7-ethyl-10-hydroxycamptothecin (SN-38), is either formed through enzymatic cleavage of CPT-11 by carboxyl esterases (CE) or through cytochrome P450 3A-mediated oxidation to 7-ethyl-10-[4-(1-piperidino)-1-amino] carbonyloxycamptothecin (NPC) and a subsequent conversion by CE. In the liver, SN-38 is glucuronidated (SN-38G) by UGT1A1, which also conjugates bilirubin. Fourteen patients were treated with 350 mg/m^2 CPT-11, and we performed pharmacokinetic analysis during a 500-h collection period. The half-life and area under the plasma concentration-time curve of SN-38 were 47±7.9 h and 2.0±0.79 μ*M*.h, respectively, both representing a 2-fold increase as compared to earlier reported estimates (A. Sparreboom, *et al.*, Clin. Cancer Res., *4:* 2747-2754, 1998). As an explanation for this phenomenon, we noted substantial formation of SN-38 from CPT-11 and NPC by plasma CE, consistent with the low circulating levels of NPC observed. In addition, transport studies in Caco-2 monolayers indicated that non-glucuronidated SN-38 could cross the membrane from apical to basolateral, indicating the potential for re-circulation processes that can prolong circulation times. Interestingly, individual levels of fecal β-glucuronidase, which is known to mediate SN-38G hydrolysis, were not related to any of the SN-38 kinetic parameters (r=0.09; *P*=0.26), suggesting that this enzyme does not play a role in SN-38 re-circulation. We have also found, in contrast to earlier data, that SN-38G/SN-38 plasma concentration ratios decrease over time from ~7 (up to 50 h) to ~1 (at 500 h). This decrease could be explained by the fact that glucuronidation of SN-38 and bilirubin is increasingly competitive at lower drug levels. In addition, no evidence was found for SN-38G transport through the Caco-2 cells. Our findings indicate that untill now the circulation time of SN-38 has been underestimated. This is of crucial importance to our understanding of the clinical action of CPT-11 and for future pharmacokinetic/pharmaco-dynamic relationships.

Introduction

CPT-11[3], a water soluble derivative of camptothecin, is currently registered for use in patients with metastatic colorectal cancer refractory to 5-fluorouracil therapy as well as first line therapy, and has shown clinical activity against several other types of solid tumors [1,2]. CPT-11 itself has weak, if any, pharmacologic activity *in vitro* and is thought to exert its antitumor activity *in vivo* following enzymatic cleavage by a CE that generates the active metabolite, SN-38, which is at least 100-fold more cytotoxic than CPT-11 [3]. Peripheral converting enzyme activity in animals has been characterized in serum [4], liver [5] and the small intestine [6], and preliminary evidence indicates CE activity within the tumor as well [7-9]. SN-38 can be metabolized very efficiently further by UDP glucuronosyltransferase 1A1 [10] and 1A7 [11] to an inactive β-glucuronide derivative, SN-38G [12,13]. Another

metabolic pathway of CPT-11 consists of a cytochrome P-450 3A4 and 3A5-mediated oxidation of the bi-piperidine side-chain attached to the core structure [14,15]. The main metabolites resulting from this pathway have been identified as APC [16] and NPC [17]. Although APC has been shown to be a poor substrate of CE in *in vitro* models [16], NPC can be converted into SN-38 by liver CE and, as such, may contribute to the overall production of the pharmacologically active species [17]. We previously reported that CPT-11 is predominantly eliminated in feces following hepatobiliary and intestinal secretion, with unchanged drug as the major excretion product followed by smaller amounts of SN-38 and APC [18]. Interestingly, SN-38G concentrations in feces were very low, presumably as a result of hydrolysis of the glucuronic acid moiety by bacterial β-glucuronidases [18,19]. We hypothesized, based also on the long terminal disposition half-life and extensive biliary secretion, that CPT-11 and its metabolites can undergo enterohepatic recirculation, and that this might play a role in the variability of pharmacokinetic parameters observed earlier. In the present study, we have re-examined the plasma disposition of CPT-11 and its metabolites using an extended sampling-time period of 500 hours in a group of patients with colorectal cancer receiving the drug as single agent at a dose level of 350 mg/m^2, and performed various *in vitro* experiments to explain the observed phenomena.

Materials and Methods

Patients and Treatment

Patients with a histologically or cytologically confirmed diagnosis of colorectal cancer refractory to standard therapy or for whom other treatment options were not available, were eligible for the present study. Additional eligibility criteria included: age between 18 and 70 years; Eastern Cooperative Oncology Group (ECOG) performance status ≤1; no previous treatment with antineoplastic agents for at least 4 weeks (or 6 weeks in case of nitrosoureas or mitomycin C); no prior treatment with CPT-11 or other topoisomerase I inhibitors; adequate hematopoietic (WBC count >3.0×10^9/L, absolute neutrophil count >2.0×10^9/L, and platelet count >100×10^9/L), renal (serum creatinine concentration ≤135 μ*M* or creatinine clearance ≥60 mL/min), and hepatic function (total serum bilirubin ≤1.25 × upper normal limit, and aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) concentrations ≤3.0 × upper normal limits); and no unresolved bowel obstruction or chronic colic disease. The current clinical protocol was approved by the Rotterdam Cancer Institute Review Board, and all patients signed informed consent before study entry.

Vials that contained 40 or 100 mg of CPT-11 (as a hydrochloride trihydrate form) formulated as a concentrated sterile solution (active drug concentration, 20 mg/mL) in *d*-sorbitol and a lactic acid-sodium hydroxide buffer system of pH 3.5-4.5 were provided by Rhône-Poulenc Rorer (Antony Cedex, France). The CPT-11 dose of 350 mg/m^2 was administered as a 90-min i.v. infusion, after dilution of the pharmaceutical preparation in 250

mL of isotonic sodium chloride. In all patients, premedication consisted of ondansetron 8 mg i.v. combined with dexamethasone 10 mg i.v., administered 30 min before the start of chemotherapy.

Sample Collection and Handling

Blood samples for pharmacokinetic analysis were drawn from a vein in the arm opposite to that used for drug infusion, and collected in 10-mL glass tubes containing lithium heparin as anticoagulant. Samples were obtained at the following time points: before drug administration; at 0.5, 1, and 1.5 h during infusion; and 0.17, 0.33, 0.5, 1, 1.5, 2, 4, 5, 8.5, 24, 32, 48, 56, 196, 360, and 504 h after the end of infusion. Blood was immediately processed to plasma by centrifugation for 5 min at 2500 rpm (4°C), which was then stored at -80°C until the time of analysis (see below). A pretreatment feces sample was collected from all patients one day prior to drug administration in a polystyrene container and stored immediately at -80°C. Following thawing, these samples were homogenized individually on ice (at 0°C) to prevent enzyme degradation in one or two volumes of a 0.1-*M* sodium acetate buffer (pH 7.0), depending on the water content of the sample, using an Ultra-Turrax T25 homogenizer (IKA-Labortechnik, Dottingen, Germany). The homogenates were centrifuged for 5 min at 15,000 rpm, and the clear supernatants were diluted one-fold with 50% glycerol in water (v/v). The dilutions were stored at –80°C until analysis for β-glucuronidase activity (see below).

Drug Analysis

Pure reference standards of CPT-11 hydrochloride trihydrate (batch: KO16) and the metabolites SN-38G trifluoroacetate (batch: YEO265), NPC trifluoroacetate (batch: YEO304), and SN-38 hydrochloride (batch: LIE783) were kindly provided by Rhône-Poulenc Rorer and were used as received. Drug concentrations in plasma were determined as the total of lactone and carboxylate forms by a validated HPLC method with fluorescence detection as described previously [20]. This method was further modified, as reported, to allow determinations of SN-38 and SN-38G at the low femtomole level [21]. Because of straying fluorescence characteristics, an incomplete extraction, and the compound's low relative retention on the reversed-phase HPLC column (Hypersil ODS with 5 μm particles), the unchanged parent drug could not be detected with this latter method [21].

Pharmacokinetic Analysis

Individual plasma concentrations of CPT-11 and its metabolites were fitted to a three-compartment model using the Siphar version 4.0 software package (SIMED, Créteil, France), as described (18). The rate constants of the various disposition phases and the AUC were estimated with a weighted-least squares method (weighting factor: $1/y$) using the fitted model, whereas the total plasma clearance of CPT-11 was calculated by dividing dose (expressed in mg base equivalents per squared meter of body surface area) and the observed AUC. The C_{max}

values were determined graphically (as observed values) in a concentration-time scatter plot. Metabolic ratios were calculated as defined [22], and included the relative extent of conversion (REC) of CPT-11 to SN-38 (*i.e.*, $AUC_{SN\text{-}38}/AUC_{CPT\text{-}11}$), and the relative extent of glucuronidation of SN-38 (*i.e.*, $AUC_{SN\text{-}38G}/AUC_{SN\text{-}38}$). The latter was also evaluated as a function of time after drug administration. The systemic SN-38 glucuronidation rate in individual patients was estimated by calculation of the biliary index values (23), expressed as $AUC_{CPT\text{-}11} \times (AUC_{SN\text{-}38}/AUC_{SN\text{-}38G})$.

Measurement of Fecal β-Glucuronidase Activity

The enzyme activity was determined by a miniaturized colorimetric assay using phenolphthalein glucuronic acid as an artificial substrate, based on a procedure described for β-glucuronidase in bile of mini pigs [24]. Briefly, 20 μL-sample aliquots of feces homogenate were mixed with 20 μL 0.1 *M* sodium acetate buffer (pH 7.0) containing 0.2% (w/v) bovine serum albumin and 10 μL of the same buffer in the presence of 0.03 *M* phenolphthalein glucuronic acid (Sigma-Aldrich Co., Zwijndrecht, the Netherlands), and incubated for 1 h at 37°C in a shaking water bath. The enzymatic reaction was terminated by the addition of 200 μL 0.1 *M* sodium phosphate buffer (pH 12.0), and the reaction product phenolphthalein was determined by measurement of the absorbance at 550 nm against a reagent blank on a Bio-Rad Model 550 automated microplate reader (Bio-Rad Laboratories, Hercules, CA). A calibration standard curve of phenolphthalein was constructed on the day of analysis, and concentration *versus* absorbance data were fitted by linear regression analysis. The mean regression equation had slope and *y* intercept values of 0.292±0.021 and −0.004±0.013 (n=15), respectively, with a Pearson's moment correlation coefficient >0.9943. Enzyme activity levels in unknown feces samples were calculated in triplicate using interpolation of the corresponding regression analysis, and expressed as micrograms of phenolphthalein liberated per hour at 37°C per milligrams of feces based on dry-weight measurements (μg/h/mg). A formal method validation was performed as described [25] by replicate analysis of quality control samples spiked to contain 3 different concentrations on several occasions in the presence of a duplicate 7-point calibration curve and reference samples containing 1.6 units/h of lyophilized type IX-A β-glucuronidase (EC 3.2.1.31) from *Escherichia coli* (Sigma, St. Louis, MO) dissolved in 50% glycerol in 0.1 *M* sodium acetate buffer (v/v). The within-run and between-run precision, as determined by one-way ANOVA, ranged from 2.18–3.55% and 2.84–4.89% (n=22 at each of the concentrations), respectively, with a mean percentage deviation from nominal values of less than ±6.14% for phenolphthalein data and ±3.30% for the β-glucuronidase reference standard.

In Vitro Metabolism of CPT-11 and NPC

Biotransformation of CPT-11 and NPC into SN-38 was studied in freshly prepared aliquots of human plasma, following a 5-min centrifugation step at 3000 × *g* of whole blood samples obtained from healthy volunteers. Prior to incubation, plasma samples were placed in a shaking water bath at 37°C for 5 min. Aliquots of 50 μL of the lactone forms of CPT-11 and NPC (from stock solutions containing 1.00 and 1.77 mg/mL in dimethyl sulfoxide, diluted in a mixture of methanol-0.01 *M* hydrochloric acid) were then added to 450 μL plasma to yield the desired final concentrations (~0.2 to 200 μ*M*), followed by slight agitation by vortex-mixing. To determine the reaction velocity (*i.e.*, V expressed in n*M* per hour per liter of plasma), sample aliquots were taken at a fixed time interval of 24 h, which was determined in preliminary experiments to be sufficiently long to achieve steady-state (not shown), and analyzed for the presence of total SN-38 as described above for plasma samples. The Michaelis-Menten kinetics of the maximum process rate (*i.e.*, V_{max}) and the drug concentration associated with $0.5 \times V_{max}$ (*i.e.*, K_m) were determined by a nonlinear regression analysis implemented on the Number Cruncher Statistical System software package (version 5.X; Jerry Hintze, East Kaysville, UT, 1992).

Drug Transport by Caco-2 Cells

The human colon adenocarcinoma cell line Caco-2 (American Type Culture Collection, Rockville, MD) was grown as monolayers in Dulbecco's minimum essential medium containing 10% heat-inactivated fetal bovine serum, 100 μg/mL penicillin and streptomycin, and 2 m*M* freshly added L-glutamine (all from Life Technologies, Inc., Breda, the Netherlands) according to procedures recommended by the American Type Culture Collection. Cells were grown at 37°C in a humidified atmosphere in 5% CO_2/95% air as stock cultures in 75-cm^3 flasks and split at ~80% confluency using trypsin-EDTA. Caco-2 cells were then seeded at a density of 2.5×10^4 cells per insert in Transwell 12-well plates containing 1-cm^2 permeable polycarbonate inserts with a 0.4-μm pore size (Costar Corp., Cambridge, MA). The Caco-2 cells were maintained to monolayer growth by medium change every 3 days, untill use in transport experiments at 28 days after seeding. The apical side of the cell layer (insert) contained 0.5 mL, whereas the basolateral side (well) contained 1.5 mL. Transport studies were initiated with CPT-11, SN-38 and SN-38G [all dissolved in a mixture of methanol-0.01*M* hydrochloric acid (1:1, v/v) and diluted further in medium at final concentrations of 1.7 and 17 μ*M* (CPT-11), 2.5 and 25 μ*M* (SN-38), and 1.5 μ*M* (SN-38G), respectively], added to either the apical or basolateral side. The final concentration of methanol in the dosing medium was always <1%. At the end of the experiment (1, 2, 6 or 24 h continuous-exposure times), the entire apical and basolateral side solutions were collected separately in 1.5-mL polypropylene tubes (Eppendorf, Hamburg, Germany), and then centrifuged for 5 min at 15,000 rpm (4°C) to remove residual particulate matter. The

supernatants were transferred to clean tubes and immediately stored at -80°C until analysis by HPLC as described [20]. Mean transport rates were calculated as the fraction of the total drug transported after exposure.

The β-glucuronidase content in the Caco-2 cells was measured using the phenolphthalein assay as described above for feces samples (detection limit, 0.3 μg/h/mg of protein), and total protein levels with the Coomassie-brilliant blue G-250 assay (26).

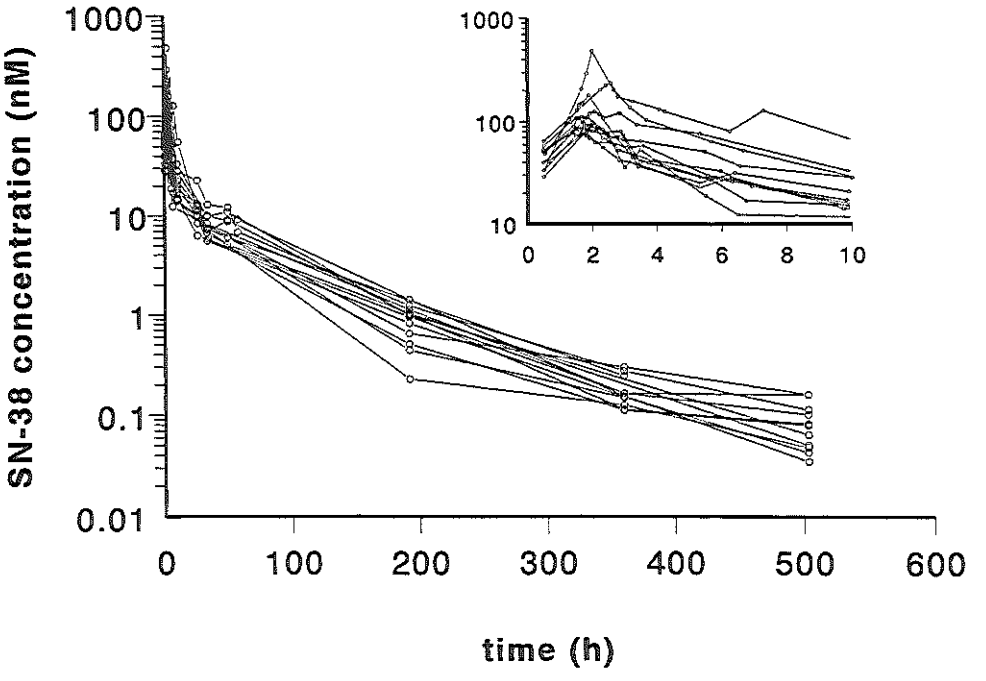


Figure 1. *Plasma concentration-time profiles of SN-38 in 14 patients treated with a 90-min i.v. infusion of CPT-11 at a dose level of 350 mg/m^2.* Inset, *concentration-time data up to 10 h after start of drug infusion.*

Results

Patient Characteristics and Toxicity

A total of 14 patients (4 females and 10 males) with various solid tumors was studied, with a median performance status of 0 (range, 0-1). The median age was 53 years (range, 37-71 years), and all patients had normal hematopoietic and liver functions at the time of the study; the median clinical chemistry values for all 14 patients included a total bilirubin level of 9 μ*M* (range, 4-12 μ*M*); a serum creatinine level of 103 μ*M* (range, 72-132 μ*M*); ASAT and ALAT levels of 34 units/L (range, 17-185 units/L) and 22 units/L (range, 6-225 units/L),

respectively; a total protein concentration of 79 g/dL (range, 69-87); and a serum albumin level of 45 g/dL (range, 38-51 g/dL). All 14 patients were assessable for complete pharmacokinetics of irinotecan and metabolites. Overall, the treatment was very well tolerated, with neutropenia being the main hematological toxicity, although neutropenia graded ≥2 (on a 4-point scale according to NCI Common Toxicity Criteria Version 2.0) was encountered in only 3 patients (20%). Gastrointestinal toxicity was most prominent among nonhematological side effects, with diarrhea graded ≥2 occurring in 8 patients (53%), and graded ≥3 in only 1 patient. One patient developed severe liver dysfunction immediately following CPT-11 administration, with total bilirubin levels rising to levels >250 μ*M* within 200 h and ASAT graded 3. This patient died eventually 9 days after CPT-11 administration after experiencing grade 4 leukocytopenia, grade 4 neutropenia, and grade 4 diarrhea. Autopsy revealed an obstructed biliary tree by pigment stones.

Table 1.
Summary of plasma pharmacokineticsa

Parameter	CPT-11	SN-38	SN-38G
C_{max} (μM)	7.53±2.20	0.168±0.115	0.639±0.258
AUC_{0-56} (μM.h)	41.7±13.9	1.12±0.570	11.0±9.26
AUC_{0-inf} (μM.h)	n.d.b	1.99±0.790	26.9±18.2
$T_{1/2}$, 0-56 (h)	13.9±3.20	29.4±18.6	26.6±9.23
$T_{1/2}$, 0-inf (h)	n.d.b	47.0±7.90	39.2±24.3
CL (L/h/m2)	15.6±4.32		
V_{ss} (L/m2)	147±55.5		
MRT (h)	10.4±2.89		
REC (AUC_{SN-38}/AUC_{CPT-11})		0.020±0.011 (range, 0.008 – 0.045)	
REG (AUC_{SN-38G}/AUC_{SN-38})		10.4±8.20 (range, 2.10 – 28.0)	
BI [$AUC_{CPT-11} \times (AUC_{SN-38}/AUC_{SN-38G})$]		2015±1359 (range, 439 – 5160)	

[a]Data were obtained from 14 patients treated with a 1.5-h i.v. infusion of CPT-11 at a dose level of 350 mg/m^2. Data are expressed as mean values ± SD.
[b] CPT-11 could not be detected in plasma samples obtained at 196, 360 and 504 h after the end of infusion with the applied HPLC assay [lower limit of quantitation, ~3.4 n*M*] (20).
Abbreviations: C_{max}, peak plasma concentration; AUC, area under the plasma concentration-time curve; $T_{1/2}$, half-life of the terminal disposition phase; CL, total plasma clearance; V_{ss}, volume of distribution at steady-state; MRT, mean residence time, REC, relative extent of conversion of CPT-11 into SN-38; REG, relative extent of glucuronidation of SN-38 into SN-38G; BI, biliary index.

Pharmacokinetics

The plasma concentration-time profiles of SN-38 after CPT-11 treatment were very similar for all patients studied (displayed in Fig. 1). In line with previous findings [18,22], plasma concentrations gradually increased to reach peak levels within 1.5 to 3 h after start of the i.v. administration, and slowly began to decline thereafter. SN-38 concentrations still remained detectable at 500 h after drug administration (lower limit of quantitation of the HPLC assay, ~13 p*M* [21]). As a result, values for $AUC_{0\text{-}inf}$ and $T_{1/2}$ were significantly higher as compared to estimates based on standard sampling time periods (Table 1). SN-38G was the principal metabolite of CPT-11 (detected in plasma of most patients), with an estimated $T_{1/2}$ slightly decreased as compared to unconjugated SN-38.

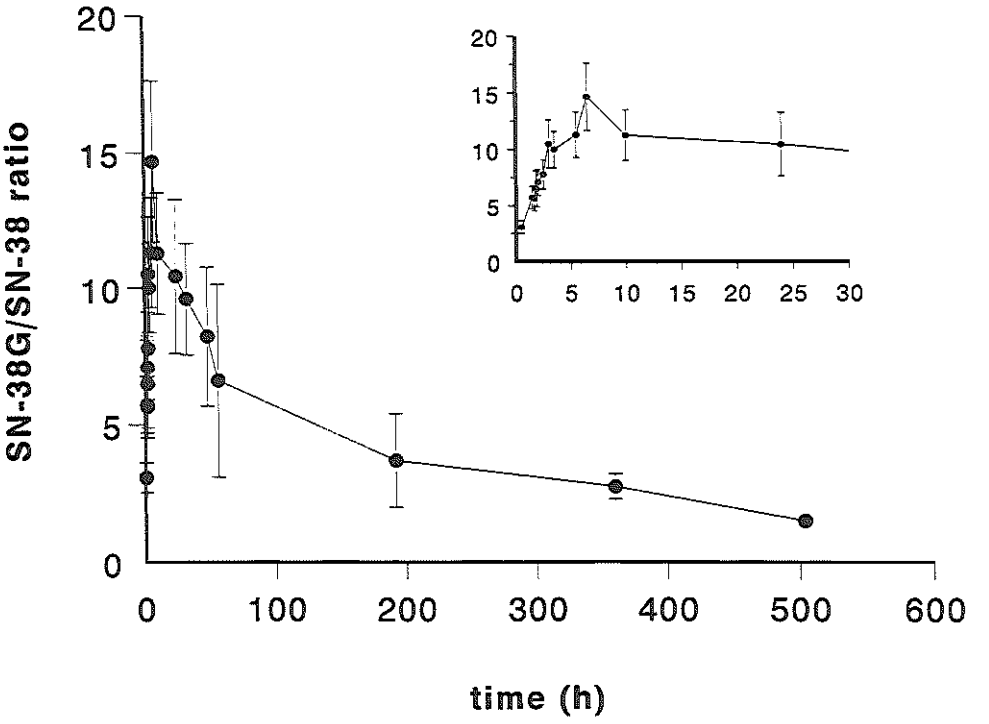


Figure 2. *Concentration ratios of SN-38G and non-glucuronidated SN-38 (SN-38G/SN-38) as a function of time after CPT-11 infusion. Data were obtained from 14 patients treated with a 90-min i.v. infusion of CPT-11 at a dose level of 350 mg/m², and are displayed as mean values (●) ± SD (error bar).* Insert, *concentration ratio-time data up to 30 h after start of infusion.*

The time profile of the molar concentration ratios of SN-38G and SN-38 was also relatively consistent between patients (Fig. 2), showing peak values at ~6 h and a gradual

decrease toward the end of the sampling time period. Data obtained from the patient with liver dysfunction and biliary obstruction showed aberrant pharmacokinetic profiles (Fig. 3), with substantially increased plasma concentrations of both SN-38G and SN-38.

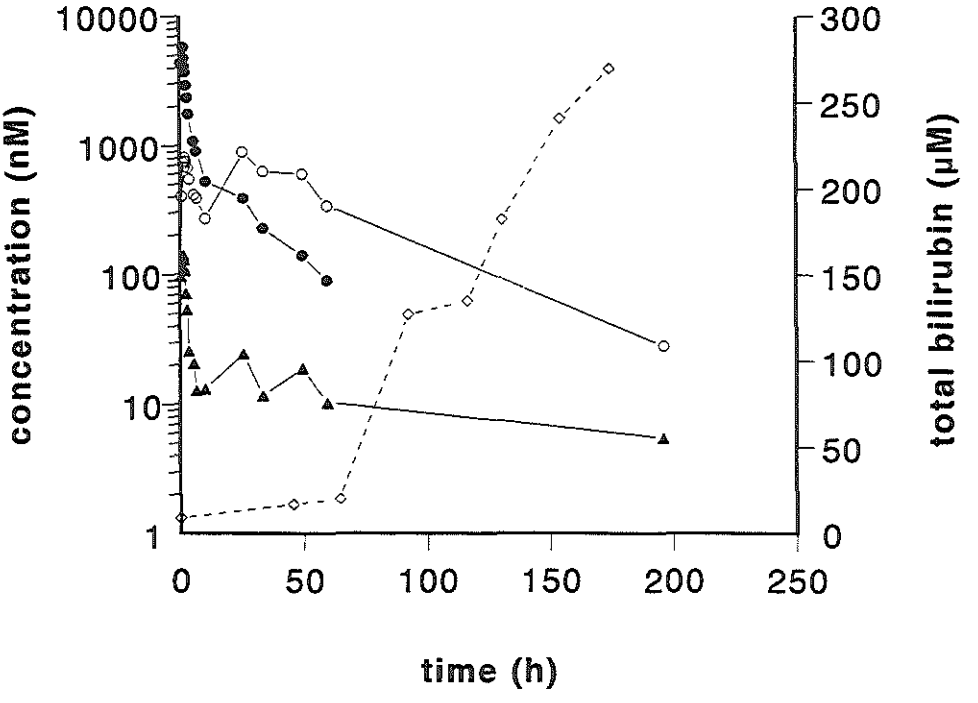


Figure 3. *Plasma concentration time profiles of CPT-11 (●), SN-38G (○), SN-38 (▲) [all left y-axis] and total bilirubin (◊, dotted line) [right y-axis] in a single patient with progressively impaired liver function treated with a 90-min i.v. infusion of CPT-11 at a dose level of 350 mg/m^2.*

In vitro Studies

Since SN-38G is susceptible to the effects of bacterial β-glucuronidases following hepatobiliary secretion [18,19], pre-therapy values of fecal β-glucuronidase activity were evaluated to gain insight into potential enterohepatic recirculation mechanisms involved in the prolonged circulation times of SN-38. Individual levels of enzyme activity were found to vary enormously between patients (range, 0.56-44.6 μg/h/mg of feces), and were not related to any of the SN-38 kinetic parameters (r=0.09; P=0.26), suggesting that this enzyme does not play a (major) role in SN-38 recirculation.

The potential of oxidative metabolites of CPT-11 taking part in the overall production of SN-38 was evaluated in experiments in freshly prepared human plasma at fixed concentrations of NPC and CPT-11 [17]. The formation of SN-38 from NPC and CPT-11 by serum CE is shown in Fig. 4. The mean values of K_m and V_{max} obtained using the Michaelis-Menten equation were 74 μ*M* and 76 pmol/h/mL plasma, respectively, which is within the same range as described for this conversion previously in an experimental setting using human liver microsomes or purified hepatic CE [17]. We also confirmed the possibility of CPT-11 transformation to SN-38 in human plasma with K_m and V_{max} values of 126 μ*M* and 52 pmol/h/mL plasma, respectively, which agree very well with data of a previous study [27].

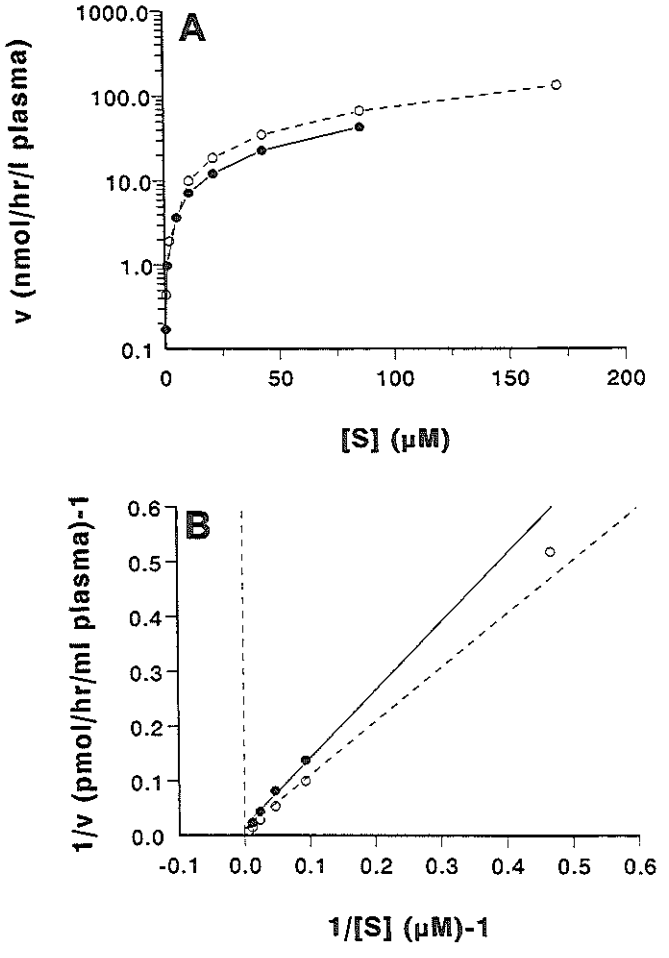


Figure 4. *Kinetics of the biotransformation of SN-38 (V, velocity) from CPT-11 (●, solid line) and NPC (○, dotted line) by human plasma as a function of the substrate (S) concentration (A), and the same data in a Michaelis-Menten plot [$V=V_{max} \times [S] / ([S] + K_m)$] (B).*

We have also studied the transepithelial flux of CPT-11, SN-38 and SN-38-G using the human colonic cell line Caco-2, a well-established model of human intestinal absorption [28-30], to further define the potential role of enterohepatic circulation in the kinetic profile of SN-38. The total transport rate was clearly time-dependent due to increased transport rates of the carboxylate forms of both compounds. CPT-11 and SN-38 were found to be transported to the apical side in a concentration-dependent manner with the 24-h exposure times, with transport rate values of 0.404 and 0.405 at low concentrations, and 0.269 and 0.399 at the highest concentrations tested, respectively. The flux from the basolateral to the apical side was substantially greater (4 to 9-fold) than that from the apical to the basolateral side. Interestingly, SN-38G could not be transported to the basolateral side following exposure at the apical side at the tested concentration. In addition, SN-38 could not be detected after exposure to SN-38G in either compartment, consistent with undetectable levels of β-glucuronidase in the Caco-2 cells (not shown).

Discussion

By prospectively measuring concentrations of CPT-11 and its metabolites in plasma of cancer patients over an extended sampling time-period of 500 h, we demonstrated in the present work that the terminal disposition half-life and AUC of the active metabolite SN-38 are ~2-fold increased as compared to estimates reported in earlier studies [18,22]. In addition, we found that the SN-38-G/SN-38 plasma concentration ratio decreases in time from ~7 (at 50 h) to ~1 (at 500 h). These data not only emphasize the need to apply appropriate kinetic models with sufficient sampling time-points for the accurate estimation of complete concentration-time profiles, but may also have direct significant clinical relevance in view of the fact that relationships between drug exposure and effect (i.e. toxicity and efficacy) are still poorly defined.

Several possible explanations can account for the increase in disposition half-life of SN-38 and the time-varying SN-38G/SN-38 concentration ratios after i.v. administration of CPT-11. Previously, biliary secretion of CPT-11, SN-38 and SN-38G has been described and quantitated in a rat model, whereas intestinal reabsorption of biliary secreted radioactivity in that same study already suggested recirculation of at least some of the compounds [31]. This, in combination with numerous clinical data, has led to the proposed enterohepatic recirculation of CPT-11 and its metabolites. It has been suggested in clinical studies that the enterohepatic recirculation is so prominent that its effects can be measured in the systemic circulation by a short rise of concentration of SN-38 at approximately 8 h after i.v. administration of CPT-11 [23]. As described in earlier reports [18,32], and again in this study, we could not confirm a consistent rise in the SN-38 plasma concentration shortly after systemic drug exposure. Direct prove of a recirculation process of any of the compounds has, to our knowledge, not yet been published. For this reason, we investigated the intestinal

absorption and transepithelial flux of CPT-11, SN-38 and SN-38-G *in vitro* in Caco-2 cell monolayers, an established model of human intestinal drug absorption resembling the small bowel. In these *in vitro* experiments, we found a time and concentration-dependent transport rate for both CPT-11 and SN-38. Although the transport rates from basolateral to apical were higher as compared to that from apical to basolateral, it is still possible that *in vivo* transport from the intestinal lumen to the circulation of CPT-11 and SN-38 is of clinical relevance, especially when intraluminal concentration of either one of the compounds is relatively high. SN-38G on the other hand could not be transported from the apical to basolateral side, consistent with its increased aqueous solubility resulting from the highly polar nature of the glucuronic acid group. In addition, the difference in absorption pattern between SN-38 and SN-38G is in accordance with the decreasing SN-38G/SN-38 ratio in time. Furthermore, we could not detect SN-38 after exposure of SN-38G in any compartment of this model, consistent with the undetectable levels of β-glucuronidase activity in the Caco-2 cells. There was also no metabolism of CPT-11 in the Caco-2 cells, in spite of the fact that cytochrome P-450 3A4, the major isoform in the human intestine [33,34], has been indicated to be present at low levels in the Caco-2 cell line [35]. To further examine the potential contribution of cytochrome P-450 3A4, if any, to the transport of CPT-11 using the Caco-2 cell system, experiments using cells with increased expression of this isozyme induced with addition of 1α,25-dihydroxyvitamin D_3 [36] to the growth medium are currently under investigation.

Since we found a concentration-dependent uptake of SN-38 in the above described model, high fecal SN-38 concentrations can be of clinical significance, in a sense that a potential recycling of SN-38 reduces the effective clearance and may add a distributional compartment by way of the enteric circuit. Many glucuronides are susceptible to the effects of enterohepatic recirculation following hydrolysis through the action of bacterial and enteric β-glucuronidases [37]. Indeed, conversion of SN-38G to SN-38 by bacterial intestinal β-glucuronidase has been shown to occur in animal models [38] as well as in humans [18]. In addition, high intraluminal concentrations of SN-38 in combination with prolonged retention and subsequent structural and functional injuries to the intestinal tract is considered to be one of the mechanisms underlying the late-onset form of intestinal toxicity encountered in patients treated with CPT-11 [39]. To assess the importance of SN-38G deconjugation with respect to potential SN-38 recycling, we therefore also evaluated the levels of β-glucuronidase activity in fecal pre-treatment specimens of individual patients. Inter-patient enzyme activity varied up to a 100-fold, and did not correlate with any of the plasma pharmacokinetic parameters of SN-38. Overall, this finding suggests that this enzyme does not play a (major) role in SN-38 recirculation. This is also consistent with our recent observation that modulation of fecal β-glucuronidase activity by neomycin co-administration has no significant influence on systemic (plasma) concentrations of SN-38 [40]. A potential approach for reducing CPT-11-induced intestinal toxicity may therefore be to reduce bacterial β-glucuronidase-mediated

deconjugation of SN-38G to limit local accumulation of SN-38 and subsequent mucosal destruction. A clinical trial to evaluate the toxicological consequences of pretreatment with neomycin before the administration of CPT-11 is in progress [4]. The paradox between the concentration-dependent absorption process of SN-38 in the Caco-2 cells and the lack of relationship of fecal β-glucuronidase activity with SN-38 pharmacokinetics is presumably caused by the lack of enzyme activity in the luminal contents of the entire small intestine where re-absorption of drug is most likely to occur [39].

Another possibility that could lead to prolongation of the terminal disposition phase of SN-38 is its continuous formation out of the oxidative metabolites of CPT-11. In an *in vitro* model, conversion of APC to SN-38 by rabbit liver CE has been described [41]. However, rabbit liver CE, though very similar to human liver CE with respect to amino acid sequence, is ~100-fold more efficient in activating APC then the human enzyme *in vitro* [42]. Indeed, *in vitro* conversion of APC to SN-38 by human CE or human liver microsomes could not be demonstrated (16), suggesting that APC is not a prodrug of SN-38 in humans. NPC on the other hand, could be metabolized into SN-38 *in vitro* by human liver microsomes and human liver CE to SN-38 following enzymatic cleavage of the 4-N-(1-piperidino)-1-amino group at C10 [17]. We have recently reported that peak plasma concentrations and AUC values of NPC are very low after CPT-11 administration [32], which could point to rapid and virtually complete conversion of this compound to SN-38 in the systemic circulation. To test this possibility, we evaluated the *in vitro* production of SN-38 from NPC and CPT-11 in freshly prepared human plasma, and found substantial formation from both compounds. These data appear to indicate that this metabolic pathway is underestimated concerning to what extent it adds to the total amount of SN-38 formed. Although this route could clearly contribute to the prolonged disposition phase of SN-38 and may be an important determinant of the substantial interpatient variability in CPT-11 pharmacokinetics observed here and elsewhere [32], the overall quantitative aspects of this source of SN-38 remain unknown.

Another potential contributing factor to the prolonged circulation time of SN-38 may come from competitive binding of SN-38 and bilirubin to UDP glucuronosyltransferases [43]. Thus, in the lower concentration regions of SN-38 competitive binding with bilirubin may inhibit glucuronidation and prolong circulation times of the active metabolite. Although we do not have direct prove for this mechanism in the clinical situation, it is known that even minor liver-enzyme disturbances and/or slight hyperbilirubinemia can give significant rise in both hematological and intestinal toxicity [44-46]. In patients with (slightly) elevated bilirubin levels, competitive binding will influence the early plasma SN-38 concentration only to a minor extent but will give a disproportional prolongation of the terminal disposition phase of SN-38 and is thus likely to affect SN-38G/SN-38 concentration ratios. The importance of this competitive interaction between SN-38 and bilirubin is further underscored by our observations made in a single patient who developed very severe toxicity. This patient developed liver failure during treatment with CPT-11 accompanied with a dramatic rise in

serum bilirubin concentrations. Compared to other patients this patient experienced at least a 10-fold increase in the SN-38 plasma concentrations, which is in accordance with this hypothesis and consistent with other published data indicating substantial increases in systemic exposure to CPT-11 and SN-38 in patients with liver dysfunction [45].

In conclusion, we have shown by applying an extended sampling-time period of 500 h that untill now the circulation time of SN-38 in cancer patients treated with CPT-11 has been greatly underestimated. Because of the poorly defined relationships between pharmacokinetic parameters and pharmacodynamic outcome of CPT-11 treatment, the presently observed prolonged terminal disposition phase of SN-38 should be taken into consideration in future studies attempting to identify kinetic correlates that would assist in prediction of both hematological and intestinal toxicity. Further investigation to reveal the clinical importance of our findings is clearly warranted.

References

1. Cunningham D, Pyrhonen S, James RD, et al. Randomized trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. Lancet. 1998;352:1413-1418.
2. Rougier P, Van Cutsem E, Bajetta E, et al. Randomised trial of irinotecan versus fluorouracil by continuous infusion after fluorouracil failure in patients with metastatic colorectal cancer. Lancet. 1998;352:1407-1412.
3. Kawato Y, Aonuma M, Hirota Y, et al. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer Res. 1991;51:4187-4191.
4. Tsuji T, Kaneda N, Kado N, et al. CPT-11 converting enzyme from rat serum: purification and some properties. J Pharmacobio-Dyn. 1991;14:341-349.
5. Rivory LP, Bowles MR, Robert J, et al. Conversion of irinotecan (CPT-11) to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), by human liver carboxylesterase. Biochem Pharmacol. 1996;52:1103-1111.
6. Zamboni WC, Houghton PJ, Thompson J, et al. Altered irinotecan and SN-38 disposition after intravenous and oral administration of irinotecan in mice bearing human neuroblastoma xenografts. Clin Cancer Res. 1998;4:455-462.
7. Danks MK, Morton CL, Pawlik CA, et al. Overexpression of a rabbit liver carboxylesterase sensitizes human tumor cells to CPT-11. Cancer Res. 1998;58:20-22.
8. Atsumi R, Okazaki O, Hakusui H. Metabolism of irinotecan to SN-38 in a tumor-isolated tumor model. Biol Pharm Bull. 1995;18:1024-1026.
9. Guichard S, Terret C, Hennebelle I, et al. CPT-11 converting carboxylesterase and topisomerase I activities in tumour and normal colon and liver tissues. Br J Cancer. 1999;80:364-370.
10. Iyer L, King CD, Whitington PF, et al. Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. J Clin Invest. 1998;101:847-854.
11. Ciotti M, Basu N, Brangi M, et al. Glucuronidation of 7-ethyl-10-hydroxycampto-thecin (SN-38) by the human UDP-glucuronosyltransferases encoded at the UGT1 locus. Biochem Biophys Res Comm. 1999;260:199-202.

12. Rivory LP, Robert J. Identification and kinetics of a β-glucuronide metabolite of SN-38 in human plasma after administration of the camptothecine derivative irinotecan. Cancer Chemother Pharmacol. 1995;36:176-179.
13. Haaz M-C, Rivory LP, Jantet S, et al. Glucuronidation of SN-38, the active metabolite of irinotecan, by human hepatic microsomes. Pharmacol Toxicol. 1997;80:91-95.
14. Lokiec F, Monegier du Sorbier B, Sanderink GJ. Irinotecan (CPT-11) metabolites in human bile and urine. Clin Cancer Res. 1996;2:1943-1949.
15. Haaz M-C, Rivory LP, Riché C, et al. Metabolism of irinotecan (CPT-11) by human hepatic microsomes: participation of cytochrome P-450 3A and drug interactions. Cancer Res. 1998;58: 468-472.
16. Rivory LP, Riou JF, Haaz M-C, et al. Identification and properties of a major plasma metabolite of irinotecan (CPT-11) isolated from the plasma of patients. Cancer Res. 1996;56:3689-3694.
17. Dodds HM, Haaz M-C, Riou JF, et al. Identification of a new metabolite of CPT-11 (irinotecan). Pharmacological properties and activation to SN-38. J Pharmacol Exp Ther. 1998;281:578-583.
18. Sparreboom A, De Jonge MJA, De Bruijn P, et al. Irinotecan (CPT-11) metabolism and disposition in cancer patients. Clin Cancer Res. 1998;4:2747-2754.
19. Schaaf L, Slatter JG, Sams J, et al. Metabolism and excretion of irinotecan (CPT-11) following IV infusion of [^{14}C]CPT-11 in patients with advanced solid tumor malignancy. Proc Am Soc Clin Oncol. 1999;19: abstract #633.
20. Sparreboom A, De Bruijn P, De Jonge MJA, et al. Liquid chromatographic determination of irinotecan and three major metabolites in human plasma, urine and feces. J Chromatogr B. 1998;712:225-235.
21. De Bruijn P, De Jonge MJA, Verweij J, et al. Femtomole quantitation of 7-ethyl-10-hydroxycamptothecine (SN-38) in plasma samples by reversed-phase high-performance liquid chromatography. Anal Biochem. 1999;269:174-178.
22. Rivory LP, Haaz MC, Canal P, et al. Pharmacokinetic interrelationships of irinotecan (CPT-11) and its three major plasma metabolites in patients enrolled in phase I/II trials. Clin Cancer Res. 1997;3:1261-1266.
23. Gupta E, Lestingi TM, Mick R, et al. Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. Cancer Res. 1994;54:3723-3725.
24. Kurtin WE, Schwesinger WH. Assay of β-glucuronidase in bile following ion-pair extraction of pigments and bile acids. Anal Biochem. 1985;147:511-516.
25. Brouwer E, Verweij J, Hauns B, et al. Linearized colorimetric assay for Cremophor EL: application to pharmacokinetics after 1-hour paclitaxel infusions. Anal Biochem. 1998;261:198-202.
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-254.
27. Gumei A, Cottrell J, Band R, et al. Human plasma irinotecan carboxylesterase converting enzyme activity in patients receiving infusional irinotecan. Proc Am Assoc Cancer Res. 1999; 40: abstract #1390.
28. Gan L-SL, Thakker DR. Applications of the Caco-2 model in the design and development of orally active drugs: elucidation of biochemical and physical barriers posed by the intestinal epithelium. Adv Drug Deliv Rev. 1997;23:77-98.
29. Lenneras H. Human jejunal effective permeability and its correlation with preclinical drug absorption models. J Pharm Pharmacol. 1997;49:627-638.
30. Barthe L, Woodley J, Houin G. Gastrointestinal absorption of drugs: methods and studies. Fundam Clin Pharmacol 1999;13:154-168.
31. Atsumi R, Suzuki W, Hakusui H. Identification of metabolites of irinotecan, a new derivative of camptothecin, in rat bile and its biliary excretion. Xenobiotica. 1991;21:1159-1169.

32. De Jonge MJA, Verweij J, De Bruijn P, et al. Pharmacokinetic, metabolic, and pharmacodynamic profiles in a dose-escalating study of irinotecan and cisplatin. J Clin Oncol. 2000;18:195-203.
33. Kolars JC, Schmiedlin-Ren P, Schuetz JD, et al. Identification of rifampin-inducible P450IIIA4 (CYP3A4) in human small bowel enterocytes. J Clin Invest. 1992;90:1871-1878.
34. Watkins PB, Wrighton SA, Schuetz EG, et al. Identification of glucocorticoid-inducible cytochromes P-450 in the intestinal mucosa of rats and man. J Clin Invest. 1987;80:1029-1036.
35. Gan L-SL, Moseley MA, Khosla B, et al. CYP3A-like cytochrome P450-mediated metabolism and polarized efflux of cyclosporin A in Caco-2 cells: Interaction between the two biochemical barriers to intestinal transport. Drug Metab Dispos. 1996;24:344-349.
36. Schmiedlin-Ren P, Thummel KE, Fischer JM, et al. Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1α,25-dihydroxy-vitamin D_3. Mol Pharmacol. 1997;51:741-754.
37. Sperker B, Backman JT, Kroemer HK. The role of beta-glucuronidase in drug disposition and drug targeting in humans. Clin Pharmacokin. 1997;33:18-31.
38. Kaneda N, Nagata H, Furuta T, et al. Metabolism and pharmaco-kinetics of the camptothecin analogue CPT-11 in the mouse. Cancer Res. 1990;50:1715-1720.
39. Takasuna K, Hagiwara T, Hirohashi M, et al. Involvement of beta-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res. 1996;56:3752-3757.
40. Sparreboom A, Kehrer D, Verweij J, et al. Pharmacokinetics of irinotecan (CPT-11) in patients treated with neomycin to diminish β-glucuronidase activity in the intestines. Clin Cancer Res. 1999;5 (Suppl. Nov.): abstract #577.
41. Guichard SM, Morton L, Krull EJ, et al. Conversion of the CPT-11 metabolite APC to SN-38 by rabbit carboxylesterase. Clin Cancer Res. 1998;4:3089-3094.
42. Danks MK, Morton CL, Krull EJ, et al. Comparison of the efficiency of CPT-11 activation by a rabbit and a human carboxylesterase for use in enzyme/prodrug therapy. Proc Am Assoc Cancer Res. 1999;40:abstract #731.
43. Wasserman E, Myara A, Lokiec F, et al. Bilirubin (bil) and SN-38 metabolism: pharmacodynamics of CPT-11 toxicity. Proc Am Soc Clin Oncol. 1998;18: abstract #714.
44. Wasserman E, Myara A, Lokiec F, et al. Severe CPT-11 toxicity in patients with Gilbert's syndrome: two case reports. Ann Oncol. 1997;8:1049-1051.
45. Raymond E, Vernillet L, Boige V, et al. Phase I and pharmacokinetic (PK) study of irinotecan (CPT-11) in cancer patients (pts) with hepatic dysfunction. Proc Am Soc Clin Oncol, 1999;19: abstract #634.
46. Gupta E, Mick R, Ramirez J, et al. Pharmacokinetic and pharmacodynamic evaluation of the topoisomerase inhibitor irinotecan in cancer patients. J Clin Oncol. 1997;15:1502-1510.

Chapter 6

Modulation of Irinotecan-Induced Diarrhea by Co-treatment with Neomycin in Cancer Patients

D.F.S. Kehrer, A. Sparreboom, J. Verweij, P. de Bruijn, C.A. Nierop, J. van de Schraaf, E.J. Ruijgrok, M.J.A. de Jonge

Department of Medical Oncology and Department of Pharmacy, Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam, The Netherlands

Clin Cancer Res, 7: 1136-1141, 2001

Abstract

This study was designed to evaluate irinotecan (CPT-11) disposition and pharmacodynamics in the presence and absence of the broad-spectrum antibiotic neomycin. Seven evaluable cancer patients experiencing diarrhea graded ≥2 after receiving CPT-11 alone (350 mg/m^2 i.v. once every three weeks) received the same dose combined with oral neomycin at 1000 mg t.i.d. (days –2 to 5) in the second course. Neomycin had no effect on the systemic exposure of CPT-11 and its major metabolites (P≥0.22). However, it changed fecal β-glucuronidase activity from 7.03±1.76 μg/h/mg (phenolphthalein assay) to undetectable levels, and decreased fecal concentrations of the pharmacologically active metabolite SN-38. Although neomycin had no significant effect on hematological toxicity (P>0.05), diarrhea ameliorated in 6 of 7 patients (P=0.033). Our findings indicate that bacterial β-glucuronidase plays a crucial role in CPT-11-induced diarrhea without affecting enterocycling and systemic SN-38 levels.

Introduction

CPT-11[3] is an inhibitor of topoisomerase I, an enzyme responsible for variations in the topological form of DNA during replication and transcription. Unlike other clinically-used camptothecin analogs, CPT-11 is a prodrug with very little inherent antitumor activity that needs to be hydrolyzed by a carboxylesterase to form the active metabolite SN-38 [1] (Fig. 1). SN-38 in its turn is efficiently metabolized by UDP glucuronosyltransferase 1A1 to form the inactive SN-38G [2].

Myelosuppression and diarrhea are among the most common side effects of CPT-11 [3], regardless of the schedule of administration. Delayed-type diarrhea is defined as diarrhea occurring >24 h after CPT-11 administration, and contrasts to the early-onset diarrhea which is acetylcholine-mediated and can be prevented by atropine [4]. CPT-11-induced delayed-type diarrhea has been reported to be severe (NCI-CTC grade 3-4) in approximately 25% of the patients [5]. Moreover even less severe diarrhea might influence continuation of therapy. The median onset of delayed-type diarrhea is day 5 after start of CPT-11 administration, and the median duration is 5 days. Delayed-type diarrhea necessitated hospitalization in 9% of the cycles for i.v. rehydration. There is no generally accepted prophylactic treatment for the delayed-type diarrhea. However, once diarrhea has occurred, a high dose loperamide regimen renders this side effect manageable [6].

Many pharmacokinetic analyses in humans have been performed in order to predict the incidence of delayed-type diarrhea, with conflicting results. Some studies reported a correlation between late-onset diarrhea and biliary secretion of the active metabolite SN-38, as determined by the extent of SN-38G measured in plasma [7].

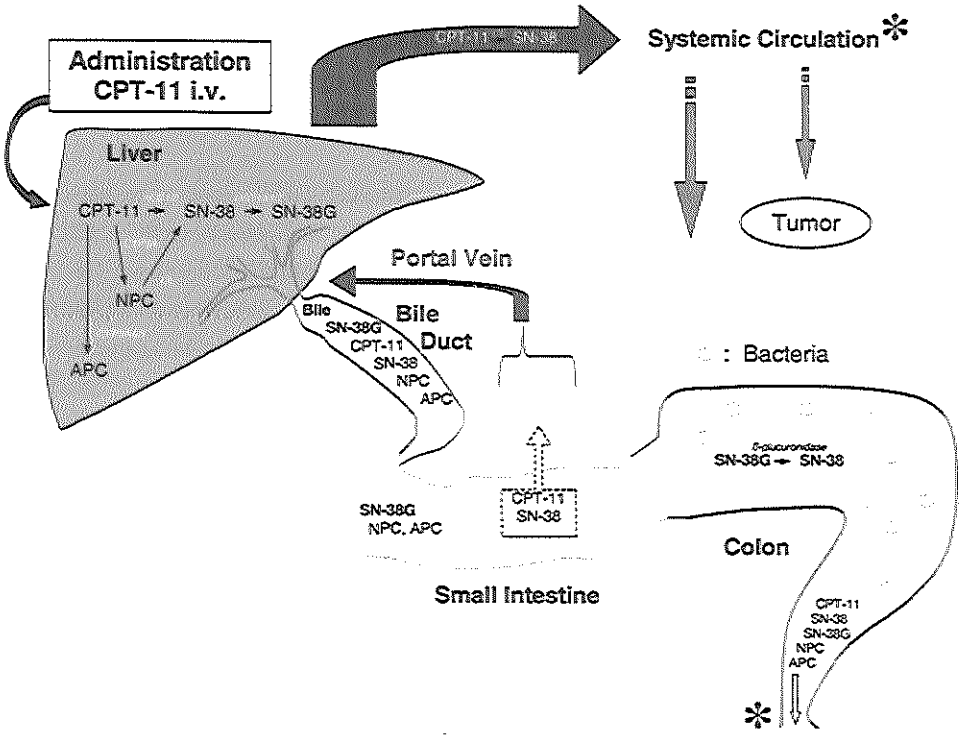


Figure 1. *Schematic representation of CPT-11 metabolism and enterohepatic recirculation.* CE, *carboxylesterase;* hCE1/2, *human carboxylesterase isoforms 1 and 2;* UGT1A1/7, *UDP glucuronosyltransferase isoforms 1A1 and 1A7;* CYP3A4/5, *cytochrome P450 isoforms 3A4 and 3A5;* APC, *7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin;* NPC, *7-ethyl-10-[4-N-(1-piperidino)-1-amino]carbonyloxycamptothecin. * indicates the sites of drug measurement.*

Recently, it was suggested from animal models that β-glucuronidases produced by microflora in the large bowel may play a major role in the development of CPT-11-induced diarrhea by mediating hydrolysis of SN-38G to form the active SN-38 [8]. Data obtained in rats have indicate that penicillin combined with streptomycin inhibited the β-glucuronidase activity from the intestinal microflora, thereby decreasing the luminal SN-38 concentration and subsequently reduce cecal damage and ameliorate diarrhea. This antibiotic treatment did not alter plasma pharmacokinetics of CPT-11 or SN-38 in the rat model [9].

Theoretically, modulation of CPT-11-induced delayed-type diarrhea in humans by co-administration of the poorly-absorbed aminoglycoside antibiotic neomycin [10] could be advantageous. In this study we assessed the influence of co-administration of oral neomycin

on the metabolic disposition and pharmacodynamics of CPT-11 in a group of cancer patients using a cross-over design.

Patients and Methods

Patients and Treatment

Patients with a histologically or cytologically confirmed diagnosis of colorectal cancer refractory to therapy with 5-fluorouracil were eligible for the present study. Additional eligibility criteria included: age between 18 and 70 years; Eastern Cooperative Oncology Group (ECOG) performance status ≤1; no previous treatment with antineoplastic agents for at least 4 weeks (or 6 weeks in case of nitrosureas or mitomycin C); no prior treatment with CPT-11 or other topoisomerase I inhibitors; adequate hematopoietic (WBC $\geq 3.0\times10^9$/L, absolute neutrophil count $\geq 2.0\times10^9$/L, and platelet count $\geq 100\times10^9$/L), renal (serum creatinine ≤135 μM or creatinine clearance ≥60 mL/min), and hepatic function (total serum bilirubin ≤1.25 × upper normal limit, and ASAT and ALAT levels ≤3.0 × upper normal limits); and no unresolved bowel obstruction or chronic colic diarrhea. The clinical protocol was approved by the Rotterdam Cancer Institute Ethics Board, and all patients signed informed consent before study entry.

Vials that contained 40 or 100 mg of CPT-11 (as a hydrochloride trihydrate form) formulated as a concentrated sterile solution (active drug concentration, 20 mg/mL) in *d*-sorbitol and a lactic acid-sodium hydroxide buffer system of pH 3.5-4.5 were provided by Aventis (Hoevelaken, The Netherlands). The CPT-11 dose of 350 mg/m^2 was administered once every three weeks as a 90-min i.v. infusion, after dilution of the pharmaceutical preparation in 250 mL of isotonic sodium chloride. In all patients, premedication consisted of ondansetron 8 mg i.v. combined with dexamethasone 10 mg i.v., both administered 30 min before the start of CPT-11 infusion. Delayed type diarrhea (in the first course) was treated with loperamide 4 mg, followed by 2 mg every 2 h for a 12-h time period after the last stool. In case the patient developed diarrhea grade 2 or higher in the first course despite loperamide therapy, neomycin (1000 mg daily × 3) was administered orally at days –2 to 5 relative to the second CPT-11 administration. All toxicities were graded according to the NCI-CTC.

Sample Collection and Analysis

Blood samples for pharmacokinetic analysis were drawn during the first course and in case of neomycin co-treatment during the second course as well, from a vein in the arm opposite to that used for drug infusion and collected in 10-mL glass tubes containing lithium heparin as anticoagulant. Samples were obtained at the following time points: before drug administration; at 0.5, 1, and 1.5 h during infusion; and 0.17, 0.33, 0.5, 1, 1.5, 2, 4, 5, 8.5, 24, 32, 48, 56, 196, 360, and 504 h after the end of infusion. Blood was immediately processed to plasma by centrifugation for 5 min at 2500 rpm (4°C), which was then stored at -80°C until

the time of analysis by high-performance liquid chromatography as described [11, 12]. A pretreatment feces sample was collected from all patients one day prior to drug administration in a polystyrene container and stored immediately at -80°C. Similarly, stool collections were obtained seperately for the duration of hospitalization (~60 h). After thawing, these samples were homogenized individually on ice (at 0°C) to prevent enzyme degradation in 1 or 2 volumes of a 0.1-M sodium acetate buffer (pH 7.0), depending on the water content of the sample, using an Ultra-Turrax T25 homogenizer (IKA-Labortechnik, Dottingen, Germany). The homogenates were centrifuged for 5 min at 15,000 rpm, and the clear supernatants were diluted one-fold with 50% glycerol in water (v/v). The dilutions were stored at –80°C until analysis for β-glucuronidase activity by a miniaturized colorimetric assay using phenolphthalein glucuronic acid as an artificial substrate [13]. Urine samples were also collected from each patient during the second treatment course to evaluate the extent of neomycin absorption, as measured by a quantitative cylinder-plate microbial assay (lower limit of detection, 1.0 μg/ml).

Feces cultures within 2 days prior to therapy were taken to determine the presence of neomycin-resistant microorganism. During the second course, feces cultures were taken daily from days –3 to 3 after CPT-11 infusion and analyzed for the presence of neomycin-resistant microorganism and/or overgrowth with Staphylococci in addition to microorganism (Salmonella, Shigella, Yersinia and Campylobacter species) and toxins (Clostridium species) that could provoke diarrhea.

Pharmacologic Analysis

Individual plasma concentrations of CPT-11 and its metabolites were fit to a three-compartment model using the Siphar version 4.0 software package (SIMED, Créteil, France), as described [14]. The rate constants of the various disposition phases and the AUC (extrapolated to infinity) were estimated with a weighted-least squares method (weighting factor: 1/y) using the model, whereas the total plasma clearance of CPT-11 was calculated by dividing dose (expressed in mg base equivalents per squared meter of body surface area) and the observed AUC. The Cmax values were determined graphically (as observed values) in a log concentration-time scattered plot. Metabolic ratios were calculated as defined (15), and included the relative extent of conversion (REC) of CPT-11 to SN-38 (i.e., AUCSN-38/AUCCPT-11), and the relative extent of glucuronidation (REG) of SN-38 (i.e., AUCSN-38G/AUCSN-38). The latter was also evaluated as a function of time after drug administration. The systemic SN-38 glucuronidation rate in individual patients was estimated by calculation of the biliary index (BI) values [7], expressed as AUCCPT-11 × (AUCSN-38/AUCSN-38G). The relative hematological toxicity, i.e. the percentage decrease in WBC, was defined as: %decrease = [(pretherapy value) – nadir value) / (pretherapy value)] × 100%.

Statistical Considerations

All pharmacokinetic parameters are reported as mean values ± SD. Since multiple measurements were performed at different times on the same patients, comparisons between the sets of observations were based on within subject differences. Therefore, variation between subjects, which is usually considerable, does not affect our ability to distinguish differences between the sets of observations, which here relate to CPT-11 courses given in the absence or presence of neomycin. The effect of neomycin on CPT-11 pharmacokinetic parameters was assessed using a paired Student's t test and the 95% confidence limits for the mean difference after testing for normality and heteroscedasticity. Similarly, the effect of neomycin on CPT-11-induced diarrhea (scored on a 4-point scale according to NCI-CTC, and treated as ordered-categorical data) was evaluated using the Wilcoxon-matched pairs signed rank sum test. All calculations were done using the NCSS 5.X Series software package (J.L. Hintze, East Kaysville, UT, 1992). Statistical significance was considered to be reached when $P<0.05$, with a two-tailed distribution.

Results

Toxicity and Pharmacodynamics

Twenty patients entered this study, of which 9 (45%) developed grade 2 diarrhea in the first treatment course and received neomycin as co-treatment in the second course. Two patients in this group were not evaluable for toxicity and pharmacokinetics in the second course; one patient went off study after the first course at his own request, and one patient was not evaluable due to early death unrelated to treatment. The 7 evaluable patients had a median age of 57 years (range, 49-71 years) and an ECOG performance state of 0-1, and all patients had normal hematopoietic and liver functions (except ASAT < 2N in two patients) at the time of study entry.

The median clinical chemistry values included a total bilirubin level of 9 μM (range, 7-11 μM); a serum creatinine level of 99 μM (range, 72-106 μM); ASAT levels of 34 units/L (range, 17-83 units/L) and ALAT levels of 22 units/L (range, 6-38 units/L); a total protein concentration of 79 g/dL (range, 69-87 g/dL); and a serum albumin level of 45 g/dL (range, 38-51 g/dL). All 7 patients experienced a grade 2 diarrhea with a median duration of 6 days (range, 5-8 days) in their first course. Of these 7 (evaluable) patients, 5 (71%, P=0.0326) did not experience any diarrhea in the second treatment course, one experienced diarrhea grade 1 (3 days) and one experienced diarrhea grade 3 (5 days) in the second course.

Table 1.

Pharmacokinetic variables of 350 mg/m2 i.v. CPT-11 for 7 patients in the absence (course 1) and presence of oral neomycin (course 2)[a]

Parameter	course 1	course 2	95% C.L. (d)	Pb
CPT-11				
C_{max} (μm)	7.67±1.90	7.62±1.72	-1.07, 1.19	0.90
$T_{1/2(z)}$ (h)	12.9±3.40	13.4±5.34	-5.99, 4.92	0.81
$AUC_{0-\infty}$ (μm·h)	41.0±15.6	45.1±20.3	-11.8, 3.52	0.22
CL (liter/h/m2)	15.8±3.79	14.8±4.42	-1.88, 3.74	0.44
V_{ss} (liter/m2)	137±36.4	141±48.9	-31.8, 22.5	0.68
MRT (h)	9.70±2.38	10.6±2.76	-2.79, 1.02	0.29
SN-38				
C_{max} (μm)	0.223±0.168	0.161±0.093	-0.081, 0.161	0.43
$T_{1/2(z)}$ (h)	46.7±6.72	41.9±3.86	-4.92, 14.4	0.25
$AUC_{0-\infty}$ (μm·h)	1.65±1.24	1.64±1.20	-0.143, 0.183	0.75
REC (×102)	2.98±2.73	2.58±2.03	-0.782, 1.70	0.36
SN-38G				
C_{max} (μm)	0.414±0.128	0.290±0.072	0.035, 0.211	0.016
$T_{1/2(z)}$ (h)	33.5±9.11	36.9±7.69	-10.1, 3.29	0.23
$AUC_{0-\infty}$ (μm·h)	8.92±4.45	8.66±3.38	-1.39, 1.90	0.69
REG	5.34±2.82	5.14±2.53	-0.476, 0.884	0.45
BI	3390±2360	3880±2830	-1260, 295	0.16

[a]Data are expressed as mean values ± SD; b Two-tailed paired Student's t test.

Abbreviations: 95% C.L. (d), 95% confidence limits for the mean difference; C_{max}, peak plasma concentration; $T_{1/2(z)}$, apparent half-life of the terminal disposition phase; $AUC_{0-\infty}$, area under the plasma concentration-time curve extrapolated to infinity; CL, total plasma clearance; V_{ss}, volume of distribution at steady-state; MRT, mean residence time, REC, relative extent of conversion of CPT-11 into SN-38 (i.e. AUC_{SN-38}/AUC_{CPT-11}); REG, relative extent of glucuronidation of SN-38 into SN-38G (i.e. AUC_{SN-38G}/AUC_{SN-38}); BI, biliary index [i.e. $AUC_{CPT-11} \times (AUC_{SN-38}/AUC_{SN-38G})$].

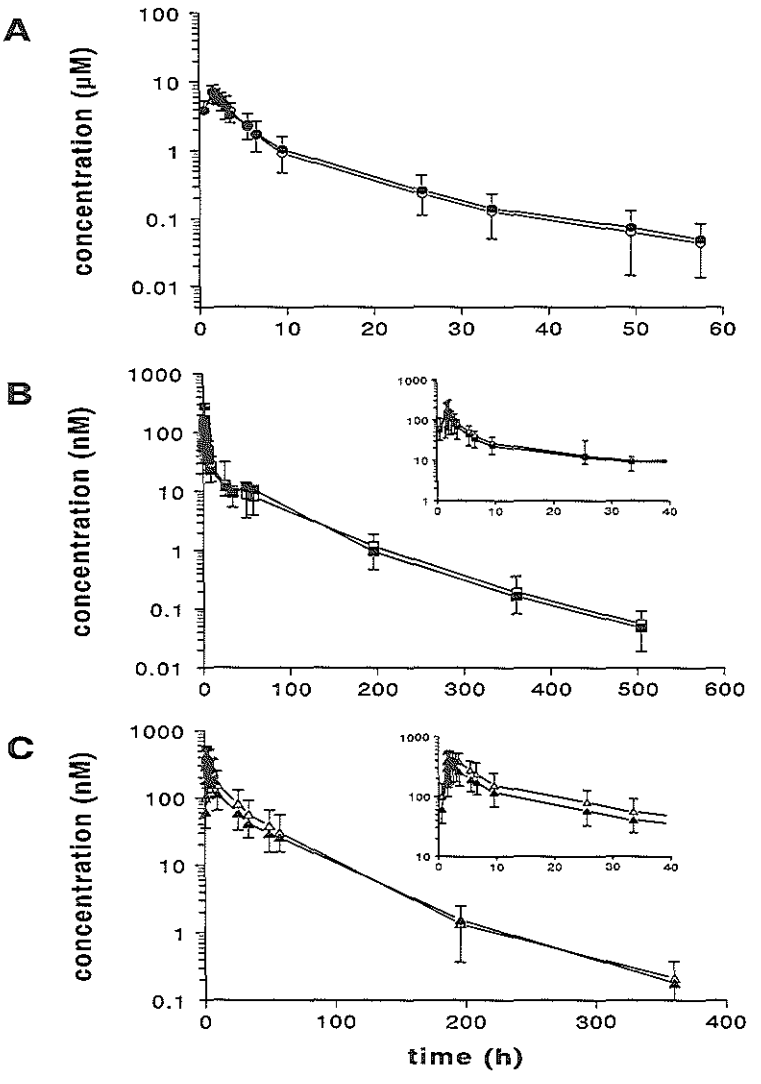


Figure 2. *Plasma concentration-time profiles of CPT-11 (A), SN-38 (B) and SN-38G (C) in patients treated with 350 mg/m² i.v. CPT-11 in the absence (○) and presence of oral neomycin (●). Data are displayed as mean values of 7 patients (○, ●) ± SD (error bar). Note the different scales used for the X-axis and Y-axis in the 3 figures.*

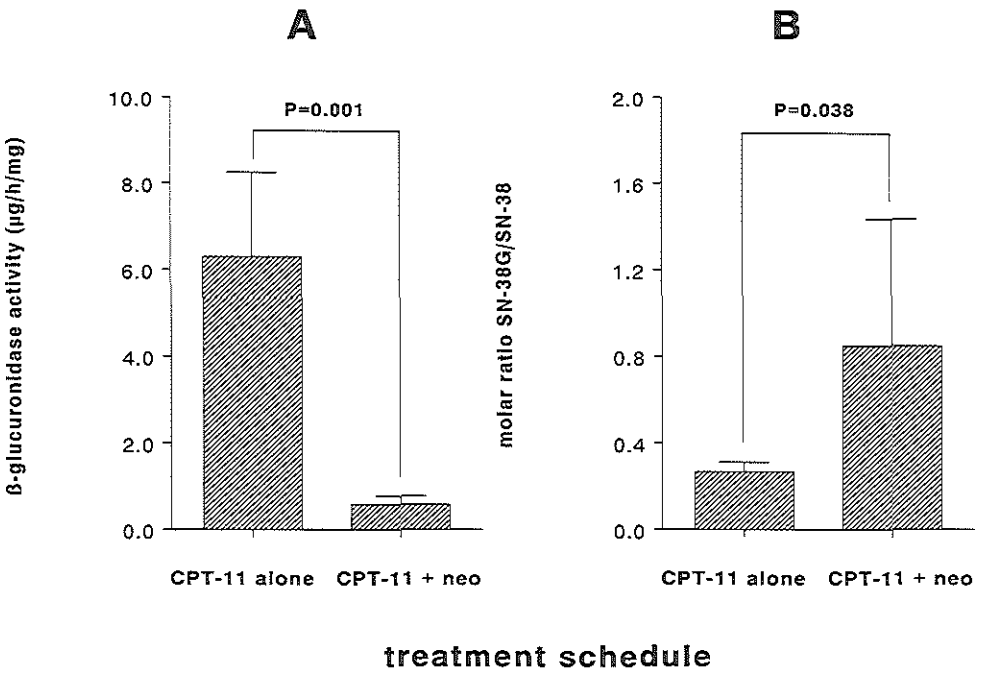


Figure 3. *Fecal β-glucuronidase activity (A) and fecal SN-38G/SN-38 concentration ratios (B) in patients treated with 350 mg/m² i.v. CPT-11 in the absence and presence of oral neomycin. Data are displayed as mean values of 7 patients (bars) ± SD (error bar).*

The relative hematological toxicity, i.e. the percentage decrease in WBC, was not significantly different between courses with values of 64.4±26.2% and 43.5±34.7% in the absence and presence of neomycin, respectively (mean difference, 20.9±5.38%; 95% confidence limits for the mean difference, 8.33, 33.5; P=0.058). The occurrence and severity of nausea or vomiting during treatment was comparable for both courses (data not shown). Feces cultures taken during the first and the second course did not reveal neomycin resistant microorganisms nor any overgrowth of pathogenic microorganisms. Cultures on infectious bacteria and toxins as mentioned above remained negative throughout treatment.

CPT-11 Pharmacokinetics

Plasma pharmacokinetics of CPT-11 and its metabolites were evaluated during both the first and second course (with neomycin) in all 7 patients (Table 1). The plasma concentration-time profiles of SN-38 after CPT-11 treatment were very similar for all patients studied (Fig. 1). Administration of neomycin did not significantly alter the AUC of CPT-11 and of the active compound SN-38, as depicted in Figs. 2A and 2B and Table 1. Only the peak levels of SN-38G were significantly lower in the second course (Fig. 2C), but this did not alter the overall exposure to this metabolite. Neomycin levels in urine were very low in all patients (range, 0.72-7.27 μg/ml; interpatient variability, 58 ± 18%), indicating no relevant ab-sorption of the drug.

Fecal β-glucuronidase activity in the first course was significantly higher than in the neomycin co-treatment course (Fig. 3A), compatible with the elimination of the intestinal microflora by neomycin. Consequently, the fecal SN-38G/SN-38 ratio was 3-fold higher in the second course (Fig. 3B).

Discussion

In the current study we obtained both clinical and pharmacokinetic data in humans which increase our insight in the pathogenesis and prevention of CPT-11-induced delayed-type diarrhea. It has been shown previously that β-glucuronidase activity derived from the intestinal microflora is able to hydrolyze biliary secreted SN-38G to the active compound SN-38 [8, 14]. SN-38 can cause histological damage to the colon with minimal damage to the small bowel as was observed in a rat model as is believed to be the main course for the occurrence of CPT-11-induced delayed type diarrhea [8]. Our present results indicate that β-glucuronidase activity can be inhibited by eliminating the microorganisms by administration of the (poorly absorbed) aminoglycoside antibiotic neomycin, without any signs of negative effects concerning bacterial overgrowth, toxins or neomycin resistance.

Since SN-38 is considered the active compound of CPT-11 treatment, it is of the utmost importance that plasma SN-38 pharmacokinetics are not altered by co-treatment with neomycin. Since SN-38 is subject to entero-hepatic recirculation [13], suppressing intestinal β-glucuronidase activity could potentially influence plasma pharmacokinetics. In this study we have shown that influencing β-glucuronidase activity by co-treatment with neomycin in patients does not alter the plasma SN-38 pharmacokinetics nor CPT-11 plasma disposition. The paradox between the entero-hepatic recirculation of SN-38 and the lack of relationship of fecal β-glucuronidase activity with SN-38 pharmacokinetics is presumably caused by the lack of enzyme activity in the luminal contents of the entire small intestine where re-absorption of drug is most likely to occur [13]. However we did find a minor but significant decline in the peak levels of SN-38G. Although the total CPT-11 metabolism has still not been completely elucidated, this particular alteration might be explained by an upregulation of serum β-

glucuronidase activity after repeated exposure to CPT-11, as has been described earlier in a rat model [16], rather than by the co-administration of neomycin. The earlier reported correlation between systemic glucuronidation and the incidence of diarrhea as expressed in the biliary index [7] could not be found in our patient population, nor in a larger previous study [17]. In contrast, we found unaltered biliary indices with and without neomycin co-treatment (Table 1), with complete disappearance of diarrhea after neomycin co-administration in 5/7 patients. This is in keeping with the postulated concept regarding the mechanism of CPT-11 induced diarrhea, being a direct local toxic effect of the active compound SN-38, and sheds light on an important mechanistic aspect of the role of bacterial β-glucuronidases in its ethiology.

In contrast to earlier data from phase III studies reporting on CPT-11-induced delayed-type diarrhea [3], only 45 % of the patients enrolled in the current study developed diarrhea in the first course of chemotherapy, and this diarrhea was never worse than grade 2. This low frequency of diarrhea could be either due to patient selection or to the small number of patients enrolled in this study. Importantly however, from the 7 patients evaluable for study purposes, 5 did not experience any diarrhea after co-administration of neomycin, while additionally in one other patient the grade of diarrhea reduced from grade 2 to 1, and this diarrhea lasted for only 3 days. Only one patient experienced a more severe diarrhea with co-administration of neomycin.

We selected the poorly absorbed aminoglycoside antibiotic neomycin because of its broad antimicrobial spectrum as well as its local gastrointestinal disposition. Indeed, neomycin concentrations in the urine were very low following its administration, indicating that no substantial drug absorption took place. As with many antibiotics, treatment with this agent can cause diarrhea and malabsorption, which could have been the case in the one patient who experienced grade 3 diarrhea in the second course. This side effect is usually seen only with chronic administration in high (≥12 g per day) dosage regimens [18]. Since neomycin can induce diarrhea, largely depending on dosage and duration of treatment, with no data available on β-glucuronidase activity compared to lengths of treatment, these are currently subject of investigation by us.

In theory, other antibiotics could also have been used in the treatment of CPT-11-induced delayed-type diarrhea, but clearly a non-resorbable drug is to be preferred. It would be even more attractive to use an agent that specifically inhibits the microbial β-glucuronidase activity. Hange-shasin-to (also referred to as TJ14) a herbal medicine that contains the β-glucuronidase inhibitor baicalin, has recently been described to be a potent inhibitor of delayed-type diarrhea caused by CPT-11 in a rat model [19] as well as in humans [20]. Unfortunately there is yet no information on possible changes in plasma β-glucuronidase activity due to this agent. Neither are there data if and how this agent influences plasma CPT-11 and SN-38 disposition, information of vital importance considering antitumor activity.

In conclusion co-treatment of CPT-11 with neomycin effectively decreases fecal β-glucuronidase activity and consequently decreases enteral SN-38 concentrations without altering the plasma pharmacokinetics and metabolic profiles of both CPT-11 and SN-38. This therapy could ultimately lead to decreased CPT-11-induced delayed-type diarrhea. A large randomized clinical trial of CPT-11 chemotherapy with or without prophylactic neomycin administration to patients is presently being conducted to confirm the present findings.

References

1. Humerickhouse R, Lohrbach K, Li L, et al. Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. Cancer Res. 2000;60:1189-1192.
2. Iyer L, King CD, Whitington PF, et al. Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. J Clin Invest. 1998;101:847-854.
3. Cunningham D, Pyrrhonen S, James RD, et al. Randomized trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. Lancet 1998;352:1413-1418.
4. Dodds H, Rivory L. The mechanism for the inhibition of acetylcholinesterases by irinotecan (CPT-11). Mol Pharmacol 1999;56:1346-53.
5. Creemers GJ, Lund B, Verweij J. Topoisomerase I inhibitors; topotecan and irinotecan. Cancer Treat Rev 1994;20:73-96.
6. Abigerges D, Armand JP, Vhabot GG, et al. Irinotecan (CPT-11) high-dose escalation using intensive high-dose loperamide to control diarrhea. J Natl Cancer Inst. 1994;86:446-449.
7. Gupta E, Lestingi TM, Mick R, et al. Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. Cancer Res. 1994;54:3723-3725.
8. Takasanu K, Hagiwara T, Hirohashi M, et al. Involvement of ß-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res. 1996;56:3752-3757.
9. Takasanu K, Hagiwara T, Hirohashi M, et al. Inhibition of intestinal microflora ß-glucuronidase modifies the distribution of the active metabolite of the antitumor agent irinotecan hydrochloride (CPT-11) in rats. Cancer Chemother Pharmacol 1998;42:280-286.
10. Goodman and Gilman, The pharmacological basis of therapeutics. 8th edition, chap 47 antimicrobial agents, neomycin, pp 1112-1113, 1990.
11. Sparreboom A, De Bruijn P, De Jonge MJA, et al. Liquid chromatographic determination of irinotecan and three major metabolites in human plasma, urine and feces. J Chromatogr B. 1998;712:225-235.
12. De Bruijn P, De Jonge MJA, Verweij J, et al. Femtomole quantitation of 7-ethyl-10-hydroxycamptothecine (SN-38) in plasma samples by reversed-phase high-performance liquid chromatography. Anal Biochem. 1999;269:174-178.
13. Kehrer D, Yamamoto W, Verweij J, et al. Factors involved in prolongation of the terminal disposition phase of SN-38: clinical and experimental studies. Clin. Cancer Res. 2000;6:3451-3458.
14. Sparreboom A, De Jonge MJA, De Bruijn P, et al. Irinotecan (CPT-11) metabolism and disposition in cancer patients. Clin Cancer Res. 1998;4:2747-2754.
15. Rivory LP, Haaz MC, Canal P, et al. Pharmacokinetic interrelationships of irinotecan (CPT-11) and its three major plasma metabolites in patients enrolled in phase I/II trials. Clin Cancer Res. 1997;3:1261-1266.

16. Kaneda N, Kurita A, Hosokawa Y, et al. Intravenous administration of irinotecan elevates the blood beta-glucuronidase activity in rats. Cancer Res. 1997;57:5305-5308.
17. De Jonge MJA, Verweij J, De Bruijn P, et al. Pharmacokinetic, metabolic, and pharmacodynamic profiles in a dose-escalating study of irinotecan and cisplatin. J Clin Oncol 2000;18:195-203.
18. Dietrich M, Gaus W, Vosse J, et al. Protective isolation and antimicrobial decontamination in patients with high susceptibility to infection. Clin Results Infection 1977;5:107.
19. Takasuna K, Kasai Y, Kitano Y, et al. Protective effects of kampo medicines and baicalin against intestinal toxicity of the new anticancer camptothecin derivate, irinotecan hydrochloride (CPT-11), in rats. Jpn J Cancer Res 1995;86:978-984.
20. Satoh H, Ishikawa H, Murakami O, et al. Reduction by hange-shashin-to (TJ-14), a herbal medicine on gastrointestinal tract complications induced by CPT-11 containing chemotherapy. Cancer Res Ther Control 1999;7:321-323.

Chapter 7

Liquid-Chromatographic Determination of Ketoconazole, a Potent Inhibitor of CYP3A4-Mediated Metabolism

P. de Bruijn, D.F.S. Kehrer, J. Verweij, A. Sparreboom

Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital, The Netherlands

J Chromatography B 753: 395-400, 2001

Abstract

A high-performance liquid chromatographic assay with UV detection has been developed for the determination of ketoconazole in human plasma. Quantitative extraction was achieved by a single solvent extraction involving a mixture of acetonitrile-*n*-butyl chloride (1:4 v/v). Ketoconazole and the internal standard (clotrimazole) were separated on a column packed with Inertsil ODS-80A material and a mobile phase composed of water-acetonitrile-tetra-hydrofuran-ammonium hydroxide-triethylamine (45:50.2:2.5:0.1:0.1, v/v). The column effluent was monitored at a wavelength of 206 nm with a detector range set at 0.5. The calibration graph was linear in the range of 20-2000 ng/ml, with a lower limit of quantitation of 20.0 ng/ml. The extraction recoveries for ketoconazole and clotrimazole in human plasma were 93±9.7% and 83±10.0%, respectively. The developed method has been successfully applied to a clinical study to examine the pharmacokinetics of ketoconazole in a cancer patient.

Introduction

There is considerable motivation for understanding drug-drug interactions with anticancer agents because of their narrow therapeutic index and the numerous concomitant medications that are administered routinely or intermittently [1]. These interactions may arise as a result of altered pharmacodynamics or pharmacokinetics of the drugs involved. In the latter case, this is usually due to modification of tissue distribution and metabolism of the drugs. Most of the data currently available to evaluate potential drug interactions with (novel) anticancer agents have been addressed by animal experiments or the use of test systems *in vitro* [1]. It has been shown that ketoconazole (Fig. 1), a synthetic imidazole-type broad-spectrum antifungal agent is a highly potent inhibitor of CYP3A4 [2,3], and can inhibit anticancer drug metabolism at concentrations as low as 1 μM [2,4]. Previous investigations have shown that with standard clinical doses of ketoconazole (200-400 mg/day p.o.), peak plasma concentrations are in the range of 4 to 20 μM [5], suggesting that concomitant treatment of ketoconazole is likely to substantially alter the disposition of various anticancer agents when administered to patients. Although the key rationale for conducting experiments *in vitro* on metabolism and drug-drug interactions is the presumed applicability to the clinical situation, for most anticancer agents there are no data yet indicating that the *in vitro* studies are predictive of results *in vivo*.

Although ketoconazole is generally well absorbed after oral administration, there is large inter- and intrasubject variation in peak concentrations after the same oral dose [6]. It has been proposed, therefore, that determination of ketoconazole concentrations in patients is expedient before attempting to draw any correlation between ketoconazole dose and biologic effects. Hence, to enable further investigation of the interaction between ketoconazole and anticancer agents, a specific and accurate assay method for the determination of ketoconazole

in human plasma was considered obligatory. Several microbiological [7-9], spectrofluorimetric [10] and electrochemical assays [8], as well as HPLC methods [11-16] have been described for the determination of ketoconazole in human plasma (using UV or fluorescence detection), but generally, these lack sufficient sensitivity. The reported HPLC methods offer greater precision than the microbiological assays, but are no more sensitive and involve complicated (solid-phase) extraction procedures. In this report, we describe a novel HPLC method with UV detection for the determination of ketoconazole in human plasma, with a lower limit of quantitation of 20 ng/ml. The method involves a rapid and highly selective one-step solvent extraction, which avoids the use of expensive and time consuming solid-phase extraction techniques for sample clean up. The method has been extensively validated, and a pilot pharmacokinetic study in a cancer patient receiving ketoconazole was included to investigate the suitability of the method.

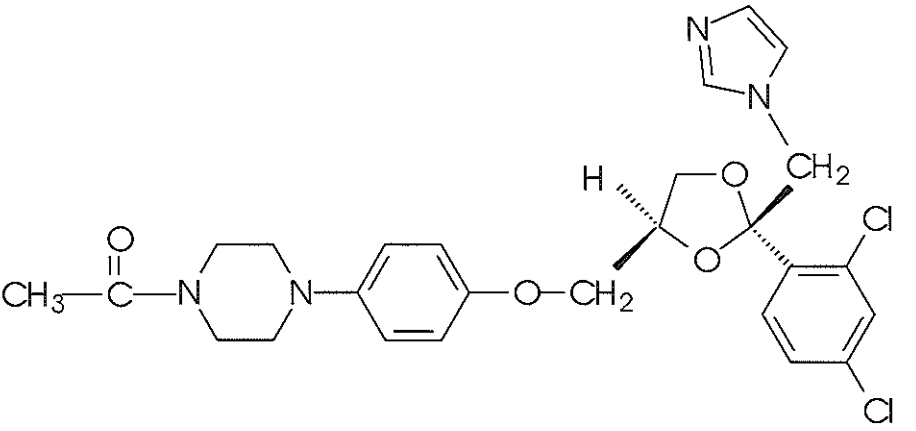


Figure 1. *Chemical structure of ketoconazole.*

Experimental

Chemicals

Ketoconazole (lot 79H4087) and the internal standard clotrimazole (lot 118H1359) were obtained from Sigma (St. Louis, MO, USA). Acetonitrile was obtained from Biosolve B.V. (Valkenswaard, The Netherlands). Tetrahydrofuran was obtained from Rathburn (Walkerburn, UK) and triethylamine and ammonium hydroxide from Baker (Deventer, The Netherlands). All chemicals and solvents were of analytical grade or better. Water was purified and deionized by the Milli-Q-UF system (Millipore, Milford, MA). Human plasma

was obtained from healthy volunteers via the Blood Bank of the University Hospital Rotterdam (Rotterdam, The Netherlands).

Preparation of Stock Solutions

Stock solutions of ketoconazole (1.0 mg/ml) and clotrimazole (1.0 mg/ml) were prepared by dissolving the appropriate amount of drug in acetonitrile and were then stored in glass at –80°C. An internal standard working solution of 10,000 ng/ml was prepared by dilution with a mixture of acetonitrile-water (1:1, v/v), and was stored for later use at 4°C.

Sample Pretreatment

A volume of 100 µl of methanol-0.05 M sodium hydroxide (40:60, v/v), 100 µl of internal standard solution (10 µg/ml) and 5 ml of acetonitrile-*n*-butyl chloride (1:4, v/v) were added to 0.5 ml of human plasma in a 12-ml glass tube and closed with a PTFE-faced screw cap. The tube was mixed for 5 min on a multi-tube vortex mixer and centrifuged for 5 min at 4000 *g*. After centrifugation, the organic layer was transferred to a clean glass tube with a Pasteur pipette, and evaporated to dryness under a constant stream of nitrogen at 60°C. A volume of 125 µl of acetonitrile-water (1:1, v/v) was added to the residue, which was reconstituted by ultrasonication for 2 min. After additional centrifugation for 2 min at 4000 *g*, the contents of the tube was transferred to a low-volume glass insert and a 100-µl aliquot was subjected to chromatography.

HPLC Analysis

The chromatographic system consisted of a ConstaMetric 3200 pump (LDC Analytical, Riviera Beach, FL, USA), a Waters 717plus autosampler operating at 4°C (Milford, MA, USA), a model SpH99 column oven (Spark Holland, Meppel, The Netherlands) and a Spectra Physics UV-2000 detector (San Jose, CA, USA). The stationary phase was composed of Inertsil ODS-80A material (5-µm particles) packed in a 150x4.6 mm I.D. stainless-steel column (GL Science, Tokyo, Japan), protected by a Lichrospher 100 RP-18 guard column (4.0x4.0 mm; 5 µm particles). The mobile phase consisted of water-acetonitrile-tetrahydrofuran-ammonium hydroxide-triethylamine (45:50.2:2.5:0.1:0.1, v/v), with the pH adjusted to 6.0 (formic acid). The flow rate of the mobile phase was set at 1.00 ml/min, and the column effluent was monitored at an absorption wavelength of 206 nm, and a detector range of 0.5. The column temperature was maintained 30°C. Acquisition and integration of data was performed with the ChromCard data analysis system connected to an ICW chromatographic work station (Fisons, Milan, Italy) running on an IBM-compatible computer under Microsoft Windows v95. Calibration graphs were calculated by weighted ($1/x^2$) least-squares linear regression analysis of the peak area ratio of ketoconazole and the internal standard versus the drug concentration of the nominal standard using the Excel v97 software package.

Precision and Accuracy

Method validation was performed according to procedures described in detail by Shah et al. [17]. With each chromatographic validation run, duplicate calibration standards were prepared in blank human plasma by serial dilution at ketoconazole concentrations of 20.0, 50.0, 100, 500, 1000 and 2000 ng/ml. Sets of quality control (QC) samples were prepared in batch in the same manner at 75.0, 750, 1500 and 15,000 ng/ml and were analyzed in quintuplicate. The QC sample containing the highest concentration was used to investigate the effect of sample dilution and/or limited sample-volume injection. The complete validation procedure was performed on four separate days. The precision of the assay was assessed by the between-run and within-run precision. Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as classification variable. The between-groups mean square (MS_{bet}), the within-groups mean square (MS_{wit}), and the grand mean (GM) of the observed concentrations across run days were calculated using the NCSS package (Version 5.X, Dr. Jerry L. Hintze, East Kaysville, UT; 1992). The between-run precision (BRP) was defined as:

$$BRP = ([(MS_{bet} - MS_{wit})/n]^{0.5}) * 100\% \qquad \text{(eq. 1)}$$

where n represents the number of replicates within each validation run. The within-run precision (WRP) was calculated as:

$$WRP = [(MS_{wit})^{0.5}/GM] * 100\% \qquad \text{(eq. 2)}$$

Lower Limit of Quantitation

For determination of the lowest standard concentration in the analytical run (LLQ) with acceptable accuracy and precision, five plasma samples from five individuals were spiked at a ketoconazole concentration of 20.0 ng/ml and analyzed in quadruplicate. For the concentration to be acceptable, the percentage deviation from the nominal value of at least 80% of the samples assayed should be ≤20%, with a resulting WRP of ≤20%.

Extraction Recovery

The extraction recovery of ketoconazole was established at concentrations of 20.0, 50.0, 100, 500, 1000 and 2000 ng/ml, by comparing peak heights of samples prepared in human plasma with those prepared in a mixture of acetonitrile-water (1:1 v/v). The recovery was determined in four independent analytical runs, and expressed as a percentage.

Specificity and Selectivity

Five different human plasma samples were used to investigate the potential interference of endogenous components. In addition, the following commonly used drugs in clinical

oncology were used for potential interference with the analytical method: acetaminophen, alizapride, atropine sulfate, codeine, dexamethasone, leucovorin, loperamide, lactulose (Legendal), lorazepam, metoclopramide, morphine hydrochloride, omeprazole, paroxetine, ranitidine, and temazepam.

Stability

The stability of ketoconazole in human plasma was investigated by analysis after storage of the various QC samples at 37°C for 30 h and after three consecutive freeze-thawing cycles. After the indicated duration and storage conditions, the samples were analyzed immediately as outlined above.

Pharmacokinetic Analysis

The patient studied was a 53-year old male with a histologically confirmed diagnosis of advanced colorectal carcinoma refractory to therapy with 5-fluorouracil. The patient received chemotherapeutic treatment with irinotecan (CPT-11), and during one of the treatment courses, ketoconazole was administered orally at a dose of 200 mg (Nizoral tablets, provided by Janssen-Cilag, Tilburg, The Netherlands), and was given every 24 hours for a period of 3 days. The current experiment was approved by the Rotterdam Cancer Institute Review Board, and the patient signed informed consent before study entry.

A total of 16 blood samples (5 ml each) were obtained and collected in 10-ml glass tubes containing 143 units of lithium heparin as anticoagulant. The samples for ketoconazole and analysis were taken immediately before intake and 1, 1.15, 1.3, 1.5, 2, 3, 3.5, 4.5, 6.5, 7.5, 10, 26, 34, 50 and 68 h after dosing. Blood was immediately processed to plasma by centrifugation for 5 min at 3000 *g*, and was kept at –20°C until storage at –80 °C. Individual plasma concentration-time data of ketoconazole were analyzed by non-compartmental models using the Siphar version 4.0 software (SIMED, Créteil, France), as described [24].

Results and Discussion

Chromatography

Fig. 2 displays chromatograms of an extract of a blank human plasma sample (**A**), an extract of a plasma sample spiked with ketoconazole at a concentration of 2000 ng/ml (**B**), and an extract of a plasma sample taken 6.5 hours after ketoconazole intake and i.v. infusion of CPT-11 (100 mg/m^2) (**C**). Ketoconazole (t_R=5.9 min) and the internal standard clotrimazole (t_R=22.0 min) were well separated, and the overall chromatographic run time was established at 30 min. Several different drugs were tested for potential interference with ketoconazole and the internal standard (see: Experimental), and none of these drugs was found to give an interfering peak during the analysis around the retention time of ketoconazole or the internal standard.

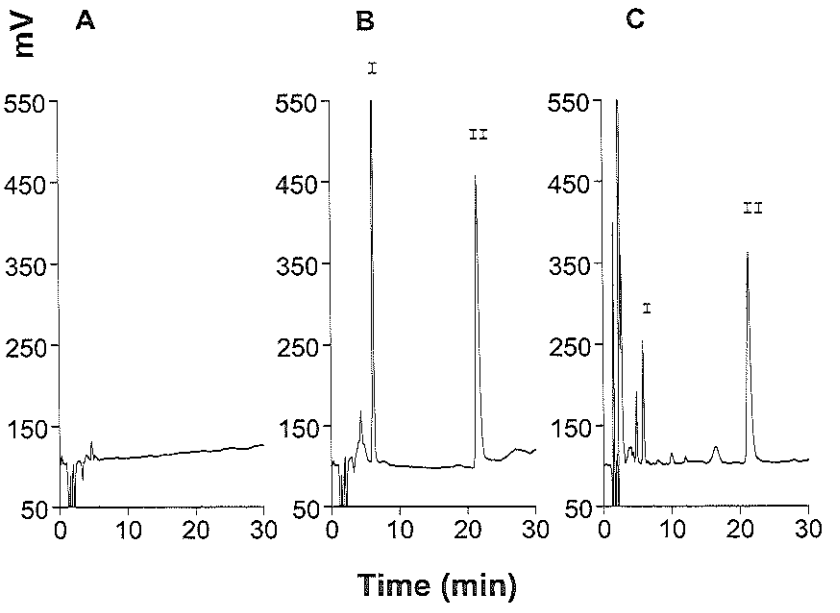


Figure 2. *Chromatograms from reversed-phase HPLC analysis of a blank human plasma sample (A), a human plasma sample spiked with ketoconazole at a concentration of 2000 ng/ml (B), and a plasma sample obtained from a male patient with colorectal cancer at 6.5 h after oral administration of ketoconazole (200 mg) (C). The labeled chromatographic peak indicte ketoconazole (I) and the internal standard clotrimazole (II), respectively.*

Validation Characteristics

The assay for ketoconazole analysis in plasma was found to be linear over the range of 20 to 2000 ng/ml, applying the peak height in combination with a weighting factor of $1/x^2$, as indicated by the mean linear-regression correlation coefficient of 0.9975 (n=4). In order to increase the sensitivity of the method, the range of the detector was set at 0.5 in stead of 1.0. In blank human plasma spiked with ketoconazole at 20 ng/ml, three out of twenty samples were outside the acceptable ±20% deviation limits, while the remaining samples were within 18%, with a mean percentage deviation from the nominal concentration and a within-run variability of 1.7 and 6.6%, respectively. Based on these results, the LLQ was established at 20 ng/ml, which is about three to four times more sensitive than earlier described methods based on HPLC [11-16].

Table 1.

Accuracy, within-run and between-run precision for the analysis of ketoconazole in spiked human plasma samples.

Nominal (ng/ml)	GM[a] (ng/ml)	ACC (%)	WRP (%)	BRP (%)	*n*
20.0	20.3	1.7	6.6	7.1	5
75.0	71.2	-5.1	10.4	0.44	5
750	739	-1.5	6.3	2.0	5
1500	1534	2.3	3.9	3.3	5
15000	16653	11.0	2.6	2.9	5

[a] Abbreviations: GM, grand mean; ACC, accuracy (percent deviation from nominal value); WRP, within-run precision; BRP, between-run precision; *n*, number of replicate observations within each validation run.

Validation data of the analytical method in terms of accuracy (percent deviation) and precision are shown in Table 1. At the upper limit of quantitation, i.e., 2000 ng/ml, the mean percentage deviation and the within-run variability were also less than 20%. The method was shown to be accurate, with an average accuracy at the four tested concentrations between -5.1 and 11.0%, and precise with a within-run and between-run precision always within 15%. In addition, sample dilution and/or limited sample-volume injection had no effect on the validation characteristics (Table 1). The mean overall extraction recoveries, determined in four separate analytical runs, were 93±9.7% for ketoconazole (n=45) and 83±10.0% for the internal standard (n=45), and were independent of the spiked concentration. Repeated freeze-thawing cycles had no influence on the stability. In addition, plasma samples spiked with ketoconazole stored for 30 h at 37°C were also stable (not shown).

Clinical Pharmacokinetics

The described analytical method was applied to a pharmacokinetic pilot study of ketoconazole given orally to a cancer patient. The observed concentration-time profile of ketoconazole is shown in Fig 3. The peak concentration of ketoconazole was 4.63 μg/ml (i.e., 8.71 μM), and the area under the concentration-time curve amounted to 12.9 μg.h/ml, which is similar to a mean (±S.D.) value of 12.3±7.7 μg.h/ml previously obtained in nine adults with hematological malignancies who were also given a dose of 200 mg [18]. The concentration-time data indicated that the ketoconazole concentration required to completely inhibit CYP3A4-mediated *in vitro* metabolism of, for example, CPT-11 (i.e., 1 μM), was achieved for at least 6 hours in the studied patient.

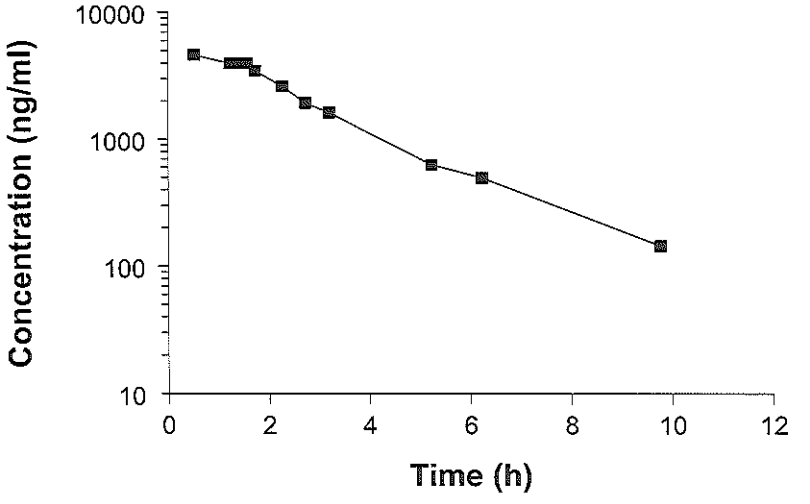


Figure 3. *Plasma concentration-time profile ketoconazole (-■-) after oral administration of a dose of 200 mg to a male cancer patient with colorectal cancer.*

In conclusion, the method presented for the determination of ketoconazole in human plasma is specific, accurate and precise, and is selective and sensitive enough to be used in clinical trials. The method permits the analysis of patient samples with low concentrations of ketoconazole, and is currently being used to investigate whether concomitant treatment of CPT-11 with ketoconazole could reduce CYP3A4-mediated drug inactivation in colorectal cancer patients.

References

1. Dresser GK, Spence JD, Bailey DG. Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. Clin Pharmacokinet. 2000;38:41-57.
2. Daneshmend TK, Warnock DW. Clinical pharmacokinetics of ketoconazole. Clin Pharmacokinet. 1988;14:13-34.
3. Venkatakrishnan K, von Moltke LL, Greenblatt DJ. Effects of the antifungal agents on oxidative drug metabolism: clinical relevance. Clin Pharmacokinet. 2000;38:111-180.
4. Santos S, Zanetta S, Cresteil T, et al. Metabolism of Irinotecan (CPT-11) by CYP3A4 and CYP3A5 in humans. Clin Cancer Res. 2000;6:2012-2020.
5 Maurice M, Pichard L, Daujat M, et al. Effects of imidazole derivatives on cytochromes P450 from human hepatocytes in primary culture. FASEB J. 1992;6:752-8.

6. Jamis-Dow CA, Pearl ML, Watkins PB, et al. Predicting drug interactions in vivo from experiments in vitro. Human studies with paclitaxel and ketoconazole. Am J Clin Oncol. 1997;20:592-599.
7. Jorgensen JH, Alexander GA, Graybill JR, et al. Sensitive bioassay for ketoconazole in serum and cerebrospinal fluid. Antimicrob Agents Chemother. 1981;20:59-62.
8. Bodet CA, Jorgensen JH, Drutz DJ. Simplified bioassay method for measurement of flucytosine or ketoconazole. J Clin Microbiol. 1985;22:157-160.
9. Turner CA, Turner A, Warnock DW. High performance liquid chromatographic determination of ketoconazole in human serum. J Antimicrob Chemother. 1986;18:757-763.
10. Khashaba PY, El-Shabouri SR, Emara KM, Mohamed AM. Analysis of some antifungal drugs by spectrophotometric and spectrofluorimetric methods in different pharmaceutical dosage forms. J Pharm Biomed Anal. 2000;22:363-376.
11. Alton KB. Determination of the antifungal agent, ketoconazole, in human plasma by high-performance liquid chromatography. J Chromatogr. 1980;221:337-344.
12. Andrews FA, Peterson LR, Beggs WH, et al. Liquid chromatographic assay of ketoconazole. Antimicrob Agents Chemother. 1981;19:110-113.
13. Pascucci VL, Bennett J, Narang PK, et al. Quantitation of ketoconazole in biological fluids using high-performance liquid chromatography. J Pharm Sci. 1983;72:1467-1469.
14. Badcock NR. Micro-determination of ketoconazole in plasma or serum by high-performance liquid chromatography. J Chromatogr. 1984;306:436-440.
15. Hoffman DW, Jones-King KL, Ravaris CL, Edkins RD. Electrochemical detection for high-performance liquid chromatography of ketoconazole in plasma and saliva. Anal Biochem. 1988;172:495-498.
16. Yuen KH, Peh KK. Simple high-performance liquid chromatographic method for determination of ketoconazole in human plasma. J Chromatogr B Biomed Sci Appl. 1998;715:436-440.
17. Shah VP, Midha KK, Dighe S, et al. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. Conference report. Eur J Drug Metab Pharmacokinet. 1991;16:249-255.
18. Stockley RJ, Daneshmend TK, Bredow MT, Warnock DW, Richardson MD, Slade RR. Ketoconazole pharmacokinetics during chronic dosing in adults with haematological malignancy. Eur J Clin Microbiol. 1986;5:513-517.

Chapter 8

Irinotecan and CYP3A4 Inhibition: Dangerous Liaisons

D.F.S. Kehrer, R.H.J. Mathijssen, J. Verweij, P. de Bruijn, A. Sparreboom

Department of Medical Oncology, Rotterdam Cancer Institute (Daniel den Hoed Kliniek) and University Hospital Rotterdam, the Netherlands

Submitted

Abstract

Background Irinotecan (CPT-11) is a prodrug of SN-38 and has been registered for the treatment of advanced colorectal cancer. It is converted by the cytochrome P450 3A4 isozyme (CYP3A4) into several inactive metabolites, including APC. To investigate the role of CYP3A4 in CPT-11 pharmacology, we evaluated the consequences of simultaneous treatment of CPT-11 with a potent enzyme inhibitor, ketoconazole, in a group of cancer patients.
Methods: A total of 7 evaluable patients was treated in a randomized cross-over design with CPT-11 (350 mg/m2 i.v. over 90 min) given alone, followed 3 weeks later by CPT-11 (100 mg/m2) in combination with ketoconazole (200 mg orally b.i.d. for 2 days), or vice versa. Serial plasma, urine and feces samples were obtained up to 500 h after dosing and analyzed for CPT-11, metabolites [SN-38, SN-38 glucuronide (SN-38G), APC], and ketoconazole by HPLC.

Results With ketoconazole co-administration, the formation of APC was reduced by 87% (P=0.002), while the relative exposure to the carboxylesterase-mediated SN-38 was increased by 110% (P=0.004). These metabolic alterations occurred without substantial changes in CPT-11 clearance (P=0.60) and formation of SN-38G (P=0.93). In spite of the reduced dose in the combination course, no significant differences in toxicity profiles were noted. Parallel in vitro studies indicated that ketoconazole had no influence on circulating carboxylesterase activity (P=0.89), or on CYP3A4-mediated SN-38 metabolism in human liver microsomes. A very minor effect was observed on the transport rate of SN-38 by Caco-2 cells, presumably mediated by inhibition of P-glycoprotein (but not BCRP) activity.

Conclusions Inhibition of CYP3A4 in cancer patients leads to significantly increased formation of SN-38 following CPT-11 administration. Simultaneous administration of various commonly prescribed drugs (either substrate or inhibitor of CYP3A4) can potentially result in fatal outcomes, and up to 4-fold reductions in CPT-11 dose are indicated.

Introduction

There is considerable motivation for understanding adverse drug interactions with anticancer agents because of their narrow therapeutic index and the numerous concomitant medications that are administered routinely or intermittently [1]. Indeed, drug interactions including those with anticancer agents are a major cause of morbidity and mortality in modern medical practice [2], causing over 100,000 deaths per year in the USA, and making it between the fourth and sixth leading cause of death in 1994 [1]. Usually, such interactions arise as a result of altered pharmacodynamics or pharmacokinetics of the drugs involved [3]. In the latter case, this is usually due to changes in metabolic routes, and several mechanisms contributing to clinically important interactions have been identified, including expression of cytochrome P450 (CYP) isozymes. This class of enzymes, particularly the CYP3A4 isozyme,

is responsible for the oxidation of over 50% of all drugs currently administered to humans [4], resulting in more polar and usually pharmacologically inactive metabolites, which can be excreted efficiently by the kidneys and the liver. It is evident that anticancer substrate drugs given in combination with drugs that are efficiently metabolized by CYP3A4, is likely to result in serious toxicity. However, most of the data currently available to evaluate possible drug interactions with anticancer agents have been addressed by animal experiments or the use of test systems in vitro [4].

In this study, we investigated the effect of CYP3A4 inhibition, using the model inhibitor ketoconazole [5], on the pharmacokinetics and toxicity profile of irinotecan (CPT-11), which is a very important drug used in the treatment of colorectal cancer [6], and a partial substrate of the CYP3A4 isozyme [7].

Materials and Methods

Patients and Treatment

Patients were eligible if they had a histologically confirmed diagnosis of metastatic colorectal cancer and proven progressive disease after first line chemotherapy (with 5-fluorouracil) or a malignancy for which there was no effective standard regimen. Additional eligibility criteria were identical as documented elsewhere [8,9]. The protocol was approved by the Ethics Board of the Rotterdam Cancer Institute, and all patients signed informed consent before study entry.

Patients screened and meeting study entry criteria were randomly assigned to receive either CPT-11 alone (as a 90-min i.v. infusion) at a dose of 350 mg/m2, followed 3 weeks later by CPT-11 at a dose of 100 mg/m2 given in combination with ketoconazole (200 mg) given orally 1 h before and 23 h after the infusion of CPT-11, or both treatment cycles were given vice versa. The 3.5-fold dose reduction of CPT-11 in combination with ketoconazole was chosen based on pharmacokinetic knowledge from earlier in vitro studies in combination with the proposed drug interaction. A restricted randomization was performed to avoid bias in cycle sequence and to keep the number of patients close for both arms. Prior to drug administration, the clinical CPT-11 formulation (Aventis Pharma, Hoevelaken, The Netherlands) was diluted in 250 ml of isotonic sodium chloride.

Patients received a standard regimen of ondansetron and dexamethasone therapy, both given 30 min prior to chemotherapy. Atropine 0.25 mg was administered subcutaneously as a prophylaxis for CPT-11-induced acute cholinergic syndrome in case the patient experienced this side effect in the previous cycle. Physical examination and toxicity assessment were performed on a weekly basis, as were clinical chemistry tests in both treatment cycles.

Pharmacologic Analysis

CPT-11, SN-38, SN-38-glucuronide (SN-38G), and APC pharmacokinetics were performed during both cycles. Blood samples (~5 ml) were collected immediately prior to

CPT-11 infusion, at 30 min after the start of the infusion, at the end of infusion, and at 10, 20 and 30 min, and 1, 1.5, 2, 4, 5, 8.5, 24, 32, 48, 56, 196 (day 8), 360 (day 15) and 500 (day 21) h post infusion. Complete urine and stool was collected in 24-h intervals for 3 days after the start of treatment in both cycles. All biologic matrices were handled as outlined [10], and CPT-11 and metabolite (SN-38, SN-38G, and APC) concentrations were determined by HPLC as described [11,12].

Pertinent pharmacokinetic parameters, including peak concentration, area under the plasma concentration-time curve (AUC), total plasma clearance (CL, defined as the ratio of dose administered in mg/m2 and AUC), the rate constant of the terminal disposition phase (k), and the half-life of the terminal disposition phase (defined as ln2/k), were calculated using a linear 3-compartment model running on Siphar v4.0 (InnaPhase, Philadelphia, PA) [13]. Metabolic ratios were calculated from plasma AUCs as well as urinary and fecal excretion data, as described previously [14]. Plasma concentrations of ketoconazole were measured by HPLC with UV detection [15].

Pharmacodynamic evaluation involved analysis of pretherapy and nadir values of white blood cell counts and absolute neutrophil counts as a function of treatment course, expressed in absolute values (in 109/liter) and in percent decrease relative to pretherapy values (e.g., [(pretherapy value – nadir value)/pretherapy value] × 100%).

Enzyme Activity in Plasma

Carboxylesterase activity in plasma samples was determined by a spectrophotometric assay using o-nitrophenyl acetate as a substrate [16], using purified carboxylesterase (EC3.1.1.1; Sigma, St. Louis, MO) as a reference. Briefly, extracts prepared by sonication in 50 mM HEPES (pH 7.4) were incubated in 3 mM of o-nitrophenyl acetate, and the absorbance at A420 was measured at 1-minute intervals for 10 minutes. Protein concentrations in extracts were determined using a Coomassie brilliant blue G250 dye-binding assay [17], using bovine serum albumin as a reference standard. Carboxylesterase activity is reported as micromoles of o-nitrophenyl acetate converted per minute per mg of protein (μmol/min/mg).

Hepatic Metabolism In Vitro

Pooled human liver microsomes (Gentest Corp., Woburn, MA) containing 1 mg of protein per ml were incubated with CPT-11 or SN-38 (both at a final concentration of 1 μM) in the presence and absence of ketoconazole (1 μM) as described [18]. Reactions were incubated for 60 min at 37°C in the presence or absence of an NADPH-regenerating system. The decrease in drug concentration was measured at serial time points by HPLC as described [19].

Drug Transport Assays

Caco-2 cells were cultured and treated as described earlier [20]. Transport studies of CPT-11 and SN-38 across complete monolayers in 12-well Transwell clusters (Costar, Cambridge, MA) with 1-cm2 polycarbonate membrane filters (0.4-μm pore size) were conducted in a controlled environment at a temperature of 37°C. The integrity of the cell monolayers was evaluated by measuring transepithelial transport of [14C]mannitol as described elsewhere [21]. Transport inhibition experiments were performed under identical conditions in the presence of ketoconazole or the P-glycoprotein/breast cancer-resistance protein (BCRP) inhibitor GF120918 (Glaxo Wellcome, Research Triangle Park, NC) added to the apical or basolateral side of the monolayer [22]. For each experiment, the mean transport rate was calculated from the linear part of the plot of the total amount of drug transported versus time. The apparent permeability coefficient (Papp), expressed in centimeters per second were calculated as $\Delta Q/\Delta t \times 1/60 \times 1/A \times 1/C0$, where $\Delta Q/\Delta t$ is the permeability rate (in μg/min), A is the surface area of the membrane (in cm2), and C0 is the initial concentration in the donor chamber (in μg/ml) [21].

Statistical Considerations

All data are presented as mean values ± standard deviation, unless stated otherwise. The statistical significance of differences in pharmacokinetic, pharmacodynamic, or in vitro data between treatments was evaluated using two-tailed, paired, Student's t tests, with a significance level of $P<0.05$, after testing for normality. All calculations were performed on the NCSS version 5.X software (J.L. Hintze, East Kaysville, UT).

Table 1.
Summary of hematological pharmacodynamics

Parameter	CPT-11	CPT-11/keto	P	Diff (±SE)*	95%C.L.
Leukocytes					
%decrease WBC	67.1±27.2 (29.9-98.0)	55.4±29.8 (17.0-91.7)	0.53	11.7±18.0	-29.8 | 53.2
Nadir (×109/L)	2.33±1.70 (0.55-4.70	3.29±1.61 (0.97-4.90)	0.38	-0.97±1.05	-3.38 | 1.44
Neutrophils					
%decrease ANC	74.5±22.5 (42.4-99.6)	56.9±23.3 (46.0-96.5)	0.26	17.6±14.5	-15.8 | 50.9
Nadir (×109/L)	1.11±0.87 (0.10-2.30)	1.93±1.19 (0.34-3.40)	0.25	-0.82±0.66	-2.33 | 0.70

Abbreviations: WBC, white blood cells; ANC, absolute neutrophil count

* Mean difference ± standard error with the 95% confidence limits.

Results

Patients and Toxicity Profiles

To determine the influence of CYP3A4 inhibition by ketoconazole on CPT-11 pharmacokinetics and toxicity, a total of 9 patients was accrued to the study. In 2 of 9 patients, ketoconazole was inadvertently only administered on day 1 in the combined schedule, and therefore these patients were considered not evaluable. Of the 7 remaining patients, 3 were male and 4 were female with a median age of 54 years (range, 42-71) and a median performance score of 1 (range, 0-2). All patients completed the study within the scheduled time without delay. The predominant disease type was colorectal cancer (n=5), and the principal toxicity consisted of neutropenia. Paired analysis of hematological pharmacodynamic parameters indicated that the degree of myelosuppression, including the percent decrease in absolute neutrophil count (P=0.26), was not significantly different between the courses, in spite of a 3.5-fold reduced CPT-11 dose when given in combination with ketoconazole (Table 1). The severity and incidence of nonhematological toxicities, including gastrointestinal side effects (nausea, vomiting and diarrhea) were also not dissimilar in the two regimens (data not shown).

CPT-11 Disposition and Effects of Ketoconazole

Since ketoconazole is a known potent inhibitor of CYP3A4, and given the prominent role of this enzyme in CPT-11 metabolism, we assessed the influence of ketoconazole on disposition profiles (Table 2). The peak concentration of ketoconazole was 4.72±2.53 μg/ml, and the AUC amounted to 15.4±9.80 μg.h/ml, which is similar to previous findings [5]. This suggests that ketoconazole concentrations required to completely inhibit CYP3A4-mediated CPT-11 metabolism in vitro (i.e., 1 μM) were achieved for at least 7 h in all patients [18]. Indeed, in the presence of ketoconazole, the formation of the CYP3A4-mediated metabolite APC was reduced by 87% (P=0.002), while the relative exposure to the pharmacologically active metabolite SN-38 increased by 110% (P=0.004). The opposing effects of these metabolic routes on drug elimination left the systemic clearance of CPT-11 almost unaffected (P=0.60). Similarly, ketoconazole had no effect on circulating levels of SN-38G (P=0.93), suggesting no effect on β-glucuronidation pathways. The cumulative exposure to the total of all metabolites, on a molar basis and normalized to a 350 mg/m2 dose, was also not dependent on the presence of ketoconazole (13.7±5.00 vs 11.5±4.50 μM.h; P=0.65). In addition, the cumulative fecal excretion of CPT-11, expressed as the percentage of the absolute dose excreted in feces within the first 56 h after drug administration, was not different in the presence or absence of ketoconazole (13.8±15.9 vs 19.5±13.9%; P=0.48). However, the recovery of SN-38 was increased by 207% and the formation of APC was reduced by 78.0%. The similarity of the terminal disposition phases in plasma of these metabolites (Fig. 1) between treatment courses indicate that the altered fecal excretion of the metabolites in the

presence of ketoconazole is related to altered formation rather than diminished biliary secretion.

Table 2.
Summary of CPT-11 pharmacokinetics (normalized to a CPT-11 dose of 350 mg/m^2)

Parameter		CPT-11	CPT-11/keto	Difference (%)	P*
Irinotecan dose (mg)**		650 (600-875)	180 (175-230)		
Infusion duration (h)**		1.50 (1.45-1.60)	1.50 (1.30-1.50)		
CPT-11					
	C_{max} (μM)	7.65±1.53	7.45±1.53		
	AUC (μM.h)	46.9±16.0	42.3±9.55		
	CL (L/h/m2)	14.9±4.51	14.7±3.16	8.94±16.5	0.60
SN-38					
	C_{max} (μM)	0.164±0.063	0.309±0.152		
	AUC (μM.h)	1.11±0.476	2.09±0.859		
	REC	0.033±0.023	0.055±0.019***	109±36.6	0.004
SN-38G					
	C_{max} (μM)	0.312±0.083	0.484±0.042		
	AUC (μM.h)	4.53±1.64	8.56±3.64		
	REG	4.18±0.372	4.14±1.17	-1.56±22.6	0.93
APC					
	C_{max} (μM)	0.627±0.283	0.139±0.049		
	AUC (μM.h)	8.03±3.80	0.843±0.0.436		
	REM	0.177±0.092	0.020±0.012	-86.9±7.14	0.002

Abbreviations: C_{max}, peak plasma concentration; AUC, area under the plasma concentration versus time curve; CL, apparent plasma clearance; REC, relative extent of conversion (AUC ratio of SN-38 to CPT-11); REG, relative extent of glucuronidation (AUC ratio of SN-38G to SN-38); REM, relative extent of metabolism (AUC ratio of APC to CPT-11).

* Wilcoxon test for matched pairs; ** Median with range; *** The coefficient sof variation of the REC values are 71.1% and 35.1%, respectively, for treatment courses with CPT-11 alone and the combination with ketoconazole.

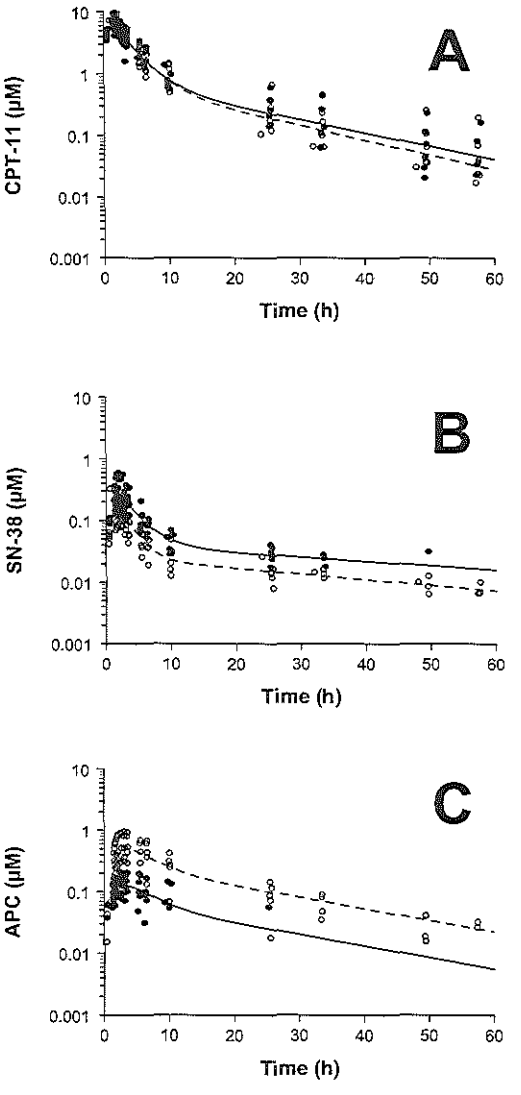


Figure 1. *Plasma concentration-time curves of CPT-11 (A), SN-38 (B) and APC (C) in the presence (solid line, ●) and absence (dashed line, ○) of ketoconazole. Data from the CPT-11/ketoconazole combination were normalized to a CPT-11 dose of 350 mg/m^2. Results are presented as all individual data time points, fitted simultaneously to a linear 3-compartment model.*

Ex Vivo Analysis of Carboxylesterase Activity in Patient Plasma

In order to gain insight into the observed altered pharmacokinetic behavior of CPT-11 in the presence of ketoconazole, various in vitro experiments were performed. We and others have shown recently that determination of plasma carboxylesterase activity by measuring

hydrolysis of an artificial substrate (such as o-nitrophenyl acetate) has utility in predicting SN-38 pharmacokinetics (8,23). The carboxylesterase activity in patient plasma, measured over 50 h after CPT-11 administration was relatively consistent, and independent of sampling time points, with mean values of 20.7±1.58 and 20.9±2.40 μmol/min/mg in the absence and presence of ketoconazole, respectively (P=0.89). This suggest that ketoconazole does not influence or induce circulating CPT-11-hydrolyzing esterases.

Effect of Ketoconazole on SN-38 Metabolism by Human Liver Microsomes

To assess a possible role of effect of ketoconazole on metabolism of SN-38 by human liver microsomes, we incubated microsomal preparations with CPT-11 and SN-38 for 60 min at 37°C. In line with previous findings [18], we found that CPT-11 was extensively metabolized to various polar compounds, including APC, but not in the presence of ketoconazole (99-100% inhibition). In contrast to previous data published in abstract form [24], no metabolic degradation by liver microsomes was observed for SN-38, and no additional effects of ketoconazole were noted (data not shown).

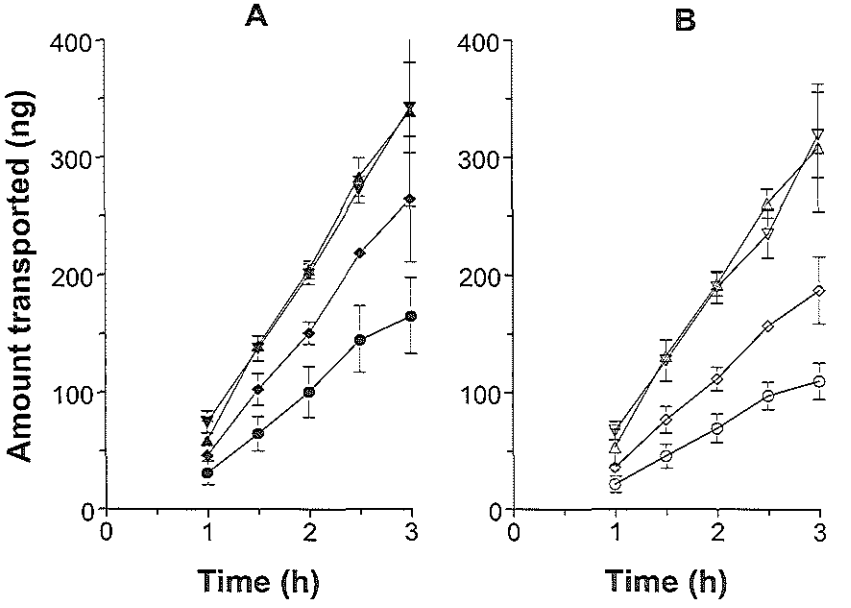


Figure 2. *Transepithelial (apical to basolateral direction) transport of CPT-11 (A) and SN-38 (B) across Caco-2 cell monolayers in the absence (●,○) and presence of ketoconazole (◆,◇), the P-glycoprotein/BCRP inhibitor GF120918 (▲,△), or ketoconazole plus GF120918 (▼,▽). Results are presented as mean values (symbol) ± standard deviation (error bar) of 3 independent observations at initial CPT-11 and SN-38 concentrations of 10 μM.*

Polarized Transport of SN-38 in Human Intestinal Cells

We next studied the transepithelial flux of CPT-11 and SN-38 in Caco-2 cell monolayers, a well-established model of human intestinal absorption [25], to further define the potential role of active transport mechanisms in SN-38 pharmacokinetics and the influence on these processes by ketoconazole. Previously, we showed that Caco-2 cells demonstrate significant expression of P-glycoprotein, multidrug-resistance associated protein (MRP-1), and the canalicular multispecific organic anion transporter (cMOAT or MRP-2) [20]. We also recently observed pronounced staining using immunoprecipitation at the apical side of Caco-2 cells with an antibody to BCRP (manuscript in preparation, A. Sparreboom and M. Maliepaard), a recently identified member of the ATP-binding cassette transporter family for which SN-38 is one of the best known substrates [26].

The flux of CPT-11 and SN-38 across Caco-2 cell monolayers, when drug was loaded on the apical side of the cells was essentially linear for up to 3 h (Fig. 2). In the presence of the P-glycoprotein/BCRP inhibitor GF120918, the Papp for the apical to basolateral transport increased more than 2-fold from 3.6×10-6 to 7.0×10-6 cm/sec and from 3.6×10-6 to 9.7×10-6 cm/sec for CPT-11 and SN-38, respectively, consistent with the known prominent role of P-glycoprotein and BCRP activity in SN-38 transport [27]. With ketoconazole, the flux of CPT-11 and SN-38 was increased by 47.2% (Papp=5.3×10-6 cm/sec) and 55.6% (Papp=5.6×10-6 cm/sec), respectively, whereas addition of ketoconazole to GF120918 had essentially no additional effect as compared to GF120918 alone (CPT-11, Papp=6.2×10-6 cm/sec; SN-38, Papp=8.0×10-6 cm/sec). Since Caco-2 cells fail to express significant CYP3A4 activity under the culture conditions employed [25], the most likely explanation for the observed effect on drug transport is inhibition of P-glycoprotein activity, a known property of ketoconazole [28].

Discussion

This study shows that inhibition of CYP3A4 by the potent enzyme inhibitor, ketoconazole, results in a substantial pharmacokinetic interaction with CPT-11. The overall results indicate a significantly reduced formation of the principal oxidative metabolite APC by 87%, as well as highly increased (approximately 110%) circulating concentrations of the pharmacologically active metabolite, SN-38. The importance of CYP3A4 activity in CPT-11 pharmacology is further substantiated by the finding that the interindividual variability in relative exposure to SN-38 was reduced from 71.1% with CPT-11 administered alone to 35.1% in the presence of ketoconazole. These data not only emphasize the need to consider the plausibility of kinetic interactions in the development of anticancer drugs, but also have a direct significant clinical relevance for treatment with CPT-11.

Clinically, the most important pathway of CPT-11 elimination consists of an esterase-mediated hydrolysis of the bipiperidine moiety, leading to SN-38 (considered essential for antitumor activity) [7]. Subsequently, UDP glucuronosyltransferase (UGT) 1A1 mediates a β-

glucuronic-acid conjugation, forming SN-38G [7], a metabolite that may play a principal role in the occurrence of CPT-11-induced diarrhea [29]. The previous recognition that CPT-11 is a substrate of CYP3A4 is a salient finding, since it makes the agent potentially subject to a host of enzyme-mediated drug interactions, even with commonly prescribed mediation [18]. For example, the prototypical CYP3A4 inhibitor troleandromycine inhibits the conversion of CPT-11 into APC in vitro almost completely [18], which is consistent with our current in vivo findings (see http://www.georgetown.edu/departments/pharmacology/davetab. html for an up to date CYP drug interaction table). In addition, both loperamide and racecadotril inhibit APC formation by more than 50%, whereas ondansetron causes inhibition by >25% [18], suggesting that some degree of interaction is to be expected with simultaneous administration of these agents with CPT-11.

The CYP3A4-mediated metabolism of CPT-11 has until now received little more than cursory interest from both pharmacologists and oncologists, most likely because the contribution of this pathway to overall elimination of CPT-11 is rather low [10], and in view of the fact that oxidative metabolism of CPT-11 is generally considered an efficient detoxification route [18]. The current observation that inhibition of CYP3A4-mediated metabolism of CPT-11 leads to an induced carboxylesterase-mediated hydrolysis to form SN-38 was rather unexpected, and in order to discriminate between increased formation and reduced elimination of SN-38 as the principal mechanism underlying this phenomenon, several additional in vitro experiments were performed. By measuring carboxylesterase activity in plasma of patients, ketoconazole was found to have no inducing effect on circulating enzyme levels that might explain the increase in relative exposure to SN-38. Similarly, ketoconazole had no effect on SN-38 biotransformation in human liver microsomes, in contrast to a previous observation suggesting prominent CYP3A4-mediated oxidation of SN-38 to a polar, currently unidentified compound [24]. Our in vitro data obtained in the Caco-2 cell monolayers suggest that ketoconazole might interfere with active drug transport mediated by P-glycoprotein, which is consistent with previous observations that ketoconazole is a (poor) inhibitor of P-glycoprotein [28]. In our patients, we observed that co-administration with ketoconazole had a marked effect on the fecal elimination of both APC and SN-38. However, the similarity of the terminal disposition phases in plasma of both metabolites between treatment courses indicates that the increased fecal excretion of SN-38 in the combination courses is unlikely related to diminished (P-glycoprotein-mediated) biliary secretion. Collectively, these findings suggest that the pharmacokinetic interference described here appears to be the result of inhibition of one of two competing enzymes involved in (hepatic) CPT-11 metabolism, which results in shunting of parent drug to SN-38.

As mentioned previously, various classes of enzymes are involved in CPT-11 metabolism, and variability in the expression of each of these will contribute to variability in drug handling between patients. In addition, this variability is further influenced by the recognition that polymorphic drug-metabolizing enzymes (e.g., UGT1A1 [30]) exist that can

alter drug disposition. Recent studies have shown that determination of individual UGT1A1 genotypes for CPT-11 treatment alone is clearly insufficient in predicting clinical effects [31]. In view of our current observation that CYP3A4-mediated metabolism of CPT-11 might be more important than held previously, we have recently initiated a prospective trial to corroborate the usefulness of gene diagnosis of UGT1A1 in combination with CYP3A4 polymorphism prior to CPT-11 chemotherapy. In addition, because CYP3A5 represents at least 50% of the total hepatic CYP3A content in people polymorphically expressing CYP3A5, this isozyme may be the most important genetic contributor to interindividual and interracial differences in CYP3A-dependent drug clearance and in responses to many agents [32]. In this context, it is of particular importance, however, that recent data indicate that the metabolism of CPT-11 by CYP3A5 was markedly different because, in contrast to CYP3A4, no APC was formed, whereas a new metabolite was formed by de-ethylation of the camptothecin moiety [33].

Clinically we found no statistically significant difference in hematologic toxicity and non-hematologic toxicity between the treatment courses, in spite of the reduced CPT-11 dose in the presence of ketoconazole, which might be explained by the increased exposure to SN-38. Previously, the idea of intentionally adding ketoconazole to systemic treatment with CYP3A4 substrate drugs (e.g. cyclosporin A) for the purpose of decreasing toxicity and costs through a reduction in dosage regimens has been put forward [34]. However, before taking advantage of the metabolic interaction between ketoconazole and CPT-11 described here to supply the two drugs as a unique preparation for clinical use, a number of important questions need to be solved. Most importantly, it is not yet known whether systemic circulating concentrations of SN-38 have any predictive ability toward antitumor activity. In fact, recent data from a study of CPT-11 administered in combination with cyclosporin A (a competitive inhibitor of CYP3A4) and phenobarbital (an inducer of UGT1A1) suggest responses to treatment in patients with metastatic colorectal and esophageal cancer without any significant diarrhea, despite a very low exposure to SN-38 [35]. This finding supports the hypothesis that intratumoral hydrolysis of CPT-11 by carboxylesterases may be more important than plasma concentrations of SN-38 [36,37]. This suggests that the concept of using intentional CYP3A4-mediated interactions with CPT-11 therapy may substantially deteriorate overall antitumor activity.

In conclusion, ketoconazole considerably increased the plasma concentrations of the pharmacologically active CPT-11 metabolite (SN-38), as a result of inhibition of CYP3A4-mediated biotransformation. Concomitant use of CPT-11 with ketoconazole or other potent substrates or inhibitors of CYP3A4 can potentially result in fatal outcomes, and up to 4-fold reductions in CPT-11 dose are indicated.

References

1. Lazarou J, Pomeranz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. J Am Med Assoc. 1998;279:1200-1205.
2. Ratain MJ. Drug combinations: dangerous liaisons or great expectations?. Ann Oncol. 1999;10:375-376.
3. Sparreboom A, Loos WJ, De Jonge MJA, et al. Clinical trial design: Incorporation of pharmacokinetic, pharmacodynamic and pharmacogenetic principles. In: Baguley BC, Kerr DJ, editors. Anticancer Drug Development. Chapter 18, Philadelphia, (PA): Academic Press; 2001. In press.
4. Ingelman-Sundberg M, Oscarson M, McLellen RA. Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment. Trends Pharmacol Sci. 1999;20:342-349.
5. Venkatakrishnan K, Von Moltke LL, Greenblatt DJ. Effects of the antifungal agents on oxidative drug metabolism: clinical relevance. Clin Pharmacokin. 2000;38:111-180.
6. Vanhoefer U, Harstrick A, Achterrath W, et al. Irinotecan in the treatment of colorectal cancer: clinical overview. J Clin Oncol. 2001;19:1501-1518.
7. Mathijssen RHJ, Van Alphen RJ, Verweij J, et al. Clinical pharmacokinetics and metabolism of irinotecan (CPT-11). Clin Cancer Res. 2001;7: in press.
8. Kehrer DFS, Yamamoto W, Verweij J, et al. Factors involved in prolongation of the terminal disposition phase of SN-38. Clin Cancer Res. 2000;6:3451-3458.
9. Kehrer DFS, Sparreboom A, Verweij J, et al. Modulation of irinotecan-induced diarrhea by cotreatment with neomycin in cancer patients. Clin Cancer Res. 2001;7:1136-1141.
10. Sparreboom A, De Jonge MJA, De Bruijn P, et al.. Irinotecan (CPT-11) metabolism and disposition in cancer patients. Clin Cancer Res. 1998;4:2747-2754.
11. Sparreboom A, De Bruijn P, De Jonge MJA, et al. Liquid chromatographic determination of irinotecan and three major metabolites in human plasma, urine and feces. J Chromatogr B Biomed Sci Appl. 1998;712:225-235.
12. De Bruijn P, De Jonge MJA, Verweij J, et al. Femtomole quantitation of 7-ethyl-10-hydroxycamptothecine (SN-38) in plasma samples by reversed-phase high-performance liquid chromatography. Anal Biochem. 1999;269:174-178.
13. De Jonge MJA, Verweij J, De Bruijn P, et al. Pharmacokinetic, metabolic, and pharmacodynamic profiles in a dose-escalating study of irinotecan and cisplatin. J Clin Oncol. 2000;18:195-203.
14. Rivory LP, Haaz MC, Canal P, et al. Pharmacokinetic interrelationships of irinotecan (CPT-11) and its three major plasma metabolites in patients enrolled in phase I/II trials. Clin Cancer Res. 1997;3:1261-1266.
15. De Bruijn P, Kehrer DFS, Verweij J, et al. Liquid chromatographic determination of ketoconazole, a potent inhibitor of CYP3A4-mediated metabolism. J Chromatogr B Biomed Sci Appl. 2001;753:395-400.
16. Khanna R, Morton CL, Danks MK, et al. Proficient metabolism of irinotecan by a human intestinal carboxylesterase. Cancer Res. 2000;60:4725-4728.
17. Zor T, Selinger Z. Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. Anal Biochem. 1996;236:302-308.
18. Haaz MC, Rivory LP, Riche C, et al. Metabolism of irinotecan (CPT-11) by human hepatic microsomes: participation of cytochrome P-450 3A and drug interactions. Cancer Res. 1998;58: 468-472.
19. De Bruijn P, Verweij J, Loos WJ, et al. Determination of irinotecan (CPT-11) and its active metabolite SN-38 in human plasma by reversed-phase high-performance liquid chromatography with fluorescence detection. J Chromatogr B Biomed Sci Appl. 1997;698:277-285.

20. Yamamoto W, Verweij J, De Bruijn P, et al. Active transepithelial transport of irinotecan (CPT-11) and its metabolites by human intestinal Caco-2 cells. Anticancer Drugs. 2001;12:419-432.
21. Artursson P. Epithelial transport of drugs in cell culture. I. A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. J Pharm Sci. 1990;79:476-482.
22. De Bruin M, Miyake K, Litman T, et al. Reversal of resistance by GF120918 in cell lines expressing the ABC half-transporter, MXR. Cancer Lett. 1999;146:117-126.
23. Guemei AA, Cottrell J, Band R, et al. Human plasma carboxylesterase and butyrylcholinesterase enzyme activity: correlations with SN-38 pharmacokinetics during prolonged infusion of irinotecan. Cancer Chemother Pharmacol. 2001;47:283-290.
24. Shepard DR, Ramirez J, Iyer L, et al. Metabolism of SN-38 by CYP3A4 and microsomes from human liver (HLM). Proc Am Soc Clin Oncol. 1999;18: 167a (abstract).
25. Artursson P, Palm K, Luthman K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Deliv Rev. 2001;46:27-43.
26. Erlichman C, Boerner SA, Hallgren CG, et al. The HER tyrosine kinase inhibitor CI1033 enhances cytotoxicity of 7-ethyl-10-hydroxycamptothecin and topotecan by inhibiting breast cancer resistance protein-mediated drug efflux. Cancer Res. 2001;61:739-748.
27. Maliepaard M, Scheffer GL, Faneyte IF, et al. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer Res. 2001;61:3458-3464.
28. Choo EF, Leake B, Wandel C, et al. Pharmacological inhibition of P-glycoprotein transport enhances the distribution of HIV-1 protease inhibitors into brain and testes. Drug Metab Dispos. 2000;28:655-660.
29. Kehrer DFS, Soepenberg O, Loos WJ, et al. Modulation of camptothecin analogs in the treatment of cancer: a review. Anticancer Drugs. 2001;12:89-105.
30. Iyer L, King CD, Whitington PF, et al. Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. J Clin Invest. 1998;101:847-854.
31. Ando Y, Saka H, Ando M, et al. Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. Cancer Res. 2000;60:6921-6926.
32. Kuehl P, Zhang J, Lin Y, et al. Sequence diversity in CYP3A promotors and characterization of the genetic basis of polymorphic CYP3A5 expression. Nat Genet. 2001;27:383-391.
33. Santos A, Zanetta S, Cresteil T, et al. Metabolism of irinotecan (CPT-11) by CYP3A4 and CYP3A5 in humans. Clin Cancer Res. 2000;6:2012-2020.
34. Albengres E, Tillement JP. Cyclosporin and ketoconazole, drug interaction or therapeutic association? Int J Clin Pharmacol Ther Toxicol. 1992;30:555-570.
35. Ratain MJ, Innocenti F, Vogelzang NJ, et al. Modulation of irinotecan (CPT-11) toxicity and pharmacokinetics by cyclosporine and phenobarbital. Proc Am Soc Clin Oncol. 2001;20:74a (abstract).
36. Danks MK, Morton CL, Pawlik CA, et al. Overexpression of a rabbit liver carboxylesterase sensitizes human tumor cells to CPT-11. Cancer Res. 1998;58:20-22.
37. Guichard S, Terret C, Hennebelle I, et al. CPT-11 converting carboxylesterase and topoisomerase activities in tumour and normal colon and liver tissues. Br J Cancer. 1999;80:364-370.

Chapter 9

Phase I and Clinical Pharmacokinetic Trial of Irinotecan Given in Combination with the Farnesyl Transferase Inhibitor R115777

D.F.S. Kehrer, A. Sparreboom, M.J.A. de Jonge, P. de Bruijn, G. de Heus, P.A. Palmer, C.J. Bol, J. Verweij

Department of Medical Oncology, Rotterdam Cancer Institute, Rotterdam, the Netherlands; Janssen Research Foundation, Beerse, Belgium

Interim analysis

Abstract

Purpose: To investigate the feasibility and the pharmacokinetics of CPT-11 administered iv once every three weeks with the orally administered farnesyltransferase inhibitor R115777 bid in different schedules.

Patients and methods: At the first dose level R115777 was administered continuously, but starting at day 3 in the first cycle, enabling pharmacokinetic interaction assessment. In the second part of the study intermittent administration of R115777 was assessed. The dose of both drugs was escalated, for CPT-11 up to the recommended single agent dose, 350 mg/m2 once every three weeks, and for R115777 up to dose limiting toxicity. Pharmacokinetics were performed during the first two cycles.

Results: Twenty-nine patients with a median age of 52 years (range 34-75), median ECOG-PS 1 (range, 0-1), received 104 cycles. The combination was tolerated reasonably well up to the level of CPT-11 350 mg/m2 with continuous R115777 200 mg bid. The toxicities most frequently observed were leucopenia and neutropenia. The frequency and severity of nausea, vomiting and diarrhea seemed to parallel those of single agent CPT-11. At the continuous R115777 schedule, the area under the curve (AUC) of SN-38 in the second cycle was significantly higher than in the first cycle ($p=0.0211$), as was the AUC of CPT-11 ($p=0.0335$). Because of clinical and pharmacokinetic findings, the administration of R115777 was changed from continuous to intermittent dosing in the second part of the study. In contrast to the continuous R115777 dosing, the AUC of SN-38 and CPT-11 did not alter with intermittent R115777 dosing ($p=0.0758$). Fatigue was more pronounced in the patients treated in the continuous schedule than with the intermittent schedule. Currently, the combination with escalating doses of intermittent R115777 bid is under investigation.

Conclusions: Although the MTD has not yet been reached, the combination therapy of CPT-11 at its full single agent dose with either continuous R115777 200 mg bid or intermittent R115777 300 mg bid is feasible. Continuous dosing of R115777 does influence the disposition of CPT-11, although the clinical relevance of this observation has not been resolved.

Introduction

Improved understanding of the signal transduction pathways has resulted in identification of various potential therapeutic targets [1-3]. Among these are the Ras-proteins that are synthesized as cytosolic precursors that attach to the membrane to transmit signals. This attachment depends on the addition of a 15-carbon farnesyl group to Ras, which reaction is catalyzed by the enzyme farnesyltransferase [4,5]. Farnesyltransferase inhibitors (FTI), such as the nonpeptidomimetic competitive agent R115777, were specifically developed to decrease Ras processing and subsequently downregulate transduction of proliferative signals

[6-8]. Other studies have suggested that FTI's might also involve other farnesylated polypeptides including RhoB [9,10], Lamin B, and centromere-associated protein pathways. Regardless of the mechanism of action of FTI's, it is clear that R115777 has shown evidence of anti-tumor activity in vitro and in vivo [11]. Anti-tumor effect has also been observed in clinical studies with different schedules of oral R115777 [12-14].

CPT-11, a water soluble derivative of camptothecin, is currently registered for use in patients with metastatic colorectal cancer refractory to 5-fluorouracil therapy as well as first line therapy, and has shown clinical activity against several other types of solid tumors [15,16]. CPT-11 itself has weak, if any, pharmacologic activity in vitro and is thought to exert its antitumor activity in vivo following enzymatic cleavage by a carboxylesterase (CE) that generates the active metabolite, SN-38, which is at least 100-fold more cytotoxic than CPT-11 [17]. Peripheral converting enzyme activity in animals has been characterized in serum [17], liver [18] and the small intestine [19], and preliminary evidence indicates CE activity within the tumor as well [20-22]. SN-38 can be metabolized very efficiently further by UDP glucuronosyltransferase 1A1 [23] and 1A7 [24] to an inactive β-glucuronide derivative, SN-38G [25,26]. Another metabolic pathway of CPT-11 consists of a cytochrome P-450 3A4 and 3A5-mediated oxidation of the bi-piperidine side-chain attached to the core structure [27-29]. The main metabolites resulting from this pathway have been identified as APC [30] and NPC [31] and are inactive.

Given the distinctly different mode of action of R115777 and CPT-11, and the need for more efficacious treatment of (colorectal) cancer, a strong rationale exists for investigating a CPT-11 based regimen in combination with R115777. In this report we present data on tolerance and pharmacokinetics of R115777, CPT-11 and its metabolites, in patients who were treated in a phase I dose finding study with oral R115777 bid, in combination with intravenous CPT-11, given once every three weeks.

Patients and Methods

Eligibility Criteria

Patients with a histologically or cytologically confirmed diagnosis of cancer refractory to standard therapy or for whom other treatment options were not available, were eligible for the present study. Additional eligibility criteria included: age at least 18 years; Eastern Cooperative Oncology Group (ECOG) performance status ≤1 and adequate unassisted oral intake; no previous treatment with antineoplastic agents for at least 4 weeks (or 6 weeks in case of nitrosoureas or mitomycin C) and no more than one prior chemotherapy regimen of treatment for advanced disease; no prior treatment with CPT-11 or other topoisomerase I inhibitors; no known diagnosis of Gilberts syndrome; no prior extensive radiotherapy defined as >25 % of the total bone marrow; adequate hematopoietic (WBC count $>3.5\times10^9$/L, and platelet count >100×109/L), renal (serum creatinine concentration ≤1.5 x upper normal limit),

and hepatic function (total serum bilirubin ≤1.5 × upper normal limit, and aspartate aminotransferase [ASAT] and alanine aminotransferase [ALAT] concentrations ≤2.5 × upper normal limits, ≤5 x upper normal limits in case of liver metastases. The clinical protocol was approved by the Rotterdam Cancer Institute Ethics Board, and all patients signed informed consent before study entry.

On-Study and Follow-up Investigations

Patients underwent a complete physical examination at enrollment and prior to every new CPT-11 administration. At these visits, ECOG performance status and weight were recorded. Determination of hematologic parameters (i.e., full blood count with white blood cell differential) and clinical chemistry analyses (i.e., electrolytes, creatinine, calcium, random glucose, albumin, phosphate, urea, uric acid, total protein, triglycerides, total cholesterol, bilirubin, alkaline phosphatase, ALAT, ASAT and lactate dehydrogenase) were performed weekly. ECG was performed before study entry and during treatment only if clinically indicated. Ophthalmological examination was performed before study entry and after each even-numbered cycle. Tumors were assessed radiologically before patients were enrolled on the study, and after every even-numbered cycle.

Pharmaceutical Preparation and Drug Administration

Vials that contained 40 or 100 mg of CPT-11 (as a hydrochloride trihydrate form) formulated as a concentrated sterile solution (active drug concentration, 20 mg/mL) in d-sorbitol and a lactic acid-sodium hydroxide buffer system of pH 3.5-4.5 were obtained from Aventis (Hoevelaken, the Netherlands). The CPT-11 dose was administered as a 90-min i.v. infusion, after dilution of the pharmaceutical preparation in 250 mL of isotonic sodium chloride. In all patients, premedication consisted of ondansetron 8 mg i.v. combined with dexamethasone 10 mg iv, both administered 30 min before the start of chemotherapy.
R115777 was provided by Janssen Research Foundation (Beerse, Belgium) as 100 mg capsules, that were stored at a dry place between 15-25 °C. The drug intakes were seperated by intervals of 12 hours, immediately after a meal.

Study Design

This is a phase I dose finding study of R115777 in combination with CPT-11 in patients with cancer. The dose escalation schemes for R115777 and CPT-11 were defined before start of the study. R115777 was started at 300 mg bid which is the recommended single agent dose, with an escalating scheme for CPT-11. CPT-11 was administered once every three weeks and was started at 200 mg/m^2 and escalated in steps of 50 mg/m^2 to the maximum tolarable dose as a single agent, 350 mg/m^2. In case the latter dose of CPT-11 would be reached further escalation of R115777 would take place in steps of 100 mg bid.

At the first cycle the patients started with CPT-11 at day 1 and with R115777 at day three, to enable pharmacokinetic interaction assessment. R115777 was administered continuously there after. Dose escalation was performed based on toxicity and pharmacokinetics. For safety, the next dose level was not opened until at least 3 patients were assessable for the first cycle in the preceding cohort. In case only one patient developed dose-limiting toxicity (DLT), the dose level was expanded with another 3 patients to a total of 6. In case less than 2 of 6 patients experienced DLT, or no DLT was observed, the dose level for the next patient cohort was established based on both clinical toxicity and pharmacokinetic data observed at the previous dose level.

Because of unacceptable toxicity observed in the very first patient, the dose of R115777 was lowered according to the protocol to 200 mg bid, administered continuously. As described in the results paragraph the schedule of R115777 was changed by an amendment to intermittent administration, consisting of bid administration for 14 out of 21 consecutive days, starting in the first cycle at day 3. In the second and following cycles R115777 was started at day 1, concomitant with CPT-11.

Toxicity Evaluation

Toxicity was assessed by the NCIC Expanded Toxicity Criteria. Dose Limiting Toxicity (DLT) was defined as: 1) grade 4 hematologic toxicity, provided neutropenia lasted more than 7 days and/or was associated with fever. 2) Non-hematological grade 3 with the exception of untreated nausea and vomiting. 3) a treatment interruption of more than three weeks due to toxicity. Only events which occured during the first cycle of protocol treatment were taken into consideration in defining DLT.

In case DLT was reached in ≥ 2 of 3 or ≥ 2 of 6 patients, dose escalation was ceased, and the dose level defined as maximum tolerable dose (MTD). Once the MTD had been determined, intermediate dose levels could be studied. The recommended dose was defined as one dose level below the MTD.

Response Evaluation

Tumor response definitions were based on the WHO criteria (WHO handbook for reporting results of cancer treatment, WHO, Geneva, 1979).

Sample Collection and Handling.

Blood samples for pharmacokinetic analysis were drawn from a vein in the arm opposite to that used for drug infusion, and collected in 10-mL glass tubes containing lithium heparin as anticoagulant during the first and second cycle. Samples on day one were obtained at the following time points: before drug administration; at 1, and 1.5 h (during infusion); and 2, 2.5, 3, 4, 7, 11, 24, and 48 hours after the start of the drug administration . On day 8 blood samples for pharmacokinetic analysis were collected in 5-ml glass tubes containing also lithium

heparin as anticoagulant. Samples on day 8 were obtained at the following time points: prior to oral drug administration, and at 1, 2, 3, 5, 8, and 12 hours (before the next oral drug administration). Blood was immediately processed to plasma by centrifugation for 5 min at 2500 rpm (4°C), which was then stored at -80°C until the time of analysis (see below).

Analytical methods

Plasma samples were assayed for total drug forms (i.e., lactone plus carboxylate) of CPT-11 and SN-38 according to a validated reversed-phase high-performance liquid chromatographic (HPLC) method reported previously [32]. Briefly, 250-µl aliquots of plasma were acidified with 500 µl of a mixture of methanol-5% (w/v) aquesous perchloric acid, to enable simultaneous measurements of total drug. The samples were rocked on a multitube vortex-mixer for 5 min, followed by centrifugation at 24,000 × g for 5 min (4°C). The clear supernatant from the extracts was diluted with methanol-0.1 M ammonium acetate containing 10 mM tetrabutylammonium sulphate (3:7, v/v), adjusted to pH 5.3 with hydrochloric acid. The diluted extracts were transferred to limited-volume inserts, and 100-µl aliquots were injected into the HPLC system. Fluorescence detection was performed at excitation and emission wavelengths of 355 and 515 nm, respectively. The determination of each compound was based on chromatographic retention times and peak area measurements in comparison with injected standards, typically over a range of 0.5 to 200 ng/ml. Calibration cuurves were prepared in drug-free human plasma and fitted by a least-squares regression function with proportional weighting using an Excel macro. The percentage deviation from nominal values, and the inter- and intra-assay precision for each compound were always less than 12%.

Plasma samples were also analyzed for the presence of unchanged R115777 using a reversed-phase HPLC method, as described elsewhere 12. Prior to analysis, plasma samples were alkalinized using 0.1 M sodium hydroxide, and extracted with a mixture of heptane-isoamyl alcohol (9:1, v/v). The chromatographic peak of R115777 and the internal standard R121550 were detected using UV absoprion measurements at 240 nm.

Pharmacologic Data Analysis

Concentration-time data of CPT-11 were analyzed using the software package Siphar v4.0 (InnaPhase, Philadelphia, PA), by determination of slopes and intercepts of the plotted curves with a tri-exponential function, as described [33]. The area under the plasma concentration versus time curve (AUC) was estimated from time zero to infinity based on the best fit curve, and was used to calculate clearance (CL) and steady-state volume of distribution (Vss) using standard equations. The terminal disposition half-life ($T_{1/2}$) was also calculated on the basis of the best fitted curve using the same program. Pharmacokinetic data of SN-38 were determined using model-independent analysis based on the trapezoidal rule. The relative extent of conversion (REC) of CPT-11 to SN-38 was calculated for each pharmacokinetic curve using the formula $AUC_{SN\text{-}38}/AUC_{CPT\text{-}11} \times 100\%$.

Plasma concentration-time profiles of R115777 were analyzed by standard noncompartmental methods using the software package WinNonlin (Pharsight, Mountain View, CA). The following pharmacokinetic parameters were calculated: maximum plasma concentration (C_{max}), time to maximum plasma concentration (t_{max}), trough plasma concentration (C_{0h}), and area under the plasma concentration versus time curve over a 12-hour dosing interval calculated by trapezoidal summation (AUC12h). Because the pharmacokinetic profile of R115777 is biphasic, initial ($T_{1/2}$ dominant) and terminal half-lives ($T_{1/2}$ terminal) were estimated by linear regression of the log-transformed concentration versus time data. The accumulation ratio was calculated by dividing the AUC_{12h} determined on day 8 by that of day 1.

Statistical considerations

Pharmacokinetic parameters for all compounds are reported as mean values ± standard deviation. Variability in parameters between the various CPT-11 and R115777 dose levels was evaluated by one-way analysis of variance to determine potential group differences. Interpatient difference in kinetics were assessed by the coefficient of variation, expressed as the ratio of the standard deviation and the observed mean value. Potential differences in pharmacokinetic data from each patient between the two evaluable courses were assessed using a paired two-tailed Student's t-test. In addition, patients were also ranked in two cohorts with R115777 given either on an intermittent or continuous schedule, and analyzed for differences in kinetic parameters by an unpaired Student's t-test. Probability values (two-sided) of less than 0.05 were regarded as statistically significant. All statistical calculations were performed using NCSS v5.X (J.L. Hintze, East Kayesville, UT; 1992).

Results

Patients and Treatment

A total of 29 patients were eligible and entered the study. The patients in the CPT-11 350 mg/m2 and R115777 400 mg bid cohort are still under treatment and the pharmacokinetics are still being processed, and therefor not considered in the results.

The median age of the patients was 52 years (range, 34-75), and there were 16 male and 13 female patients. All patients had a ECOG-PS 0-1. The predominant tumor type was colon cancer (15) while 8 patients had other gastrointestinal tumors (esophagus, pancreas, duodenum) and 6 had Adeno Carcinoma of Unknown Primary (ACUP). Four patients received one cycle of the combination regimen only. Therefore their pharmacokinetic results are not evaluable. The number of patients at each dose level are listed in Table 1.

The first patient at the first dose level of CPT-11 200 mg/m2 and R115777 300 mg bid developed grade 3 fatigue with severe weight loss, and was admitted to the hospital for intravenous rehydration. It was decided, also in view of the toxicity profile of R115777 in

other ongoing studies, to decrease the starting dose of R115777 to 200 mg bid according to the protocol. Four dose levels of CPT-11 were explored (200, 250, 300, 350 mg/m^2) combined with fixed R115777 200 mg bid continuously administered (Table 1).

Table 1.
Dose levels

Level	CPT 11 mg/m^2	R115777 mg bid C/I*	No. patients	No. Cycles (range)
A	200	200 C	4 (1x NE)	12 (2-6)
B	250	200 C	4 (1x NE)	7 (1-4)
C	300	200 C	3	16 (2-10)
D	350	200 C	6	25 (1-6)
E	350	200 I	6	22 (2-10)
F	350	300 I	6	22 (2-5)
G	350	400 I	ongoing	Ongoing

*C = continuous
I = intermittent

During the study the administration schedule of R115777 was amended to an intermittent schedule, based on pharmacokinetic data which revealed a pharmacokinetic interaction between R115777 and CPT-11 (to be discussed below). To investigate the impact on the pharmacokinetics of the change in the schedule, it was decided that at the dose of R115777 200 mg bid and CPT-11 350 mg/m^2, both the continuous and the intermittent administration were to be explored, although no formal DLT was observed in the continuous dosing schedule. However, continuous dosing in subsequent cycles seemed impossible with this schedule, since in three patients treated with continuous R115777 at this level, subsequent doses of CPT-11 had to be reduced because of delayed recovery from hematological toxicity. Further escalation of R115777 was pursued in the intermittent schedule only, with 300 mg bid and 400 mg bid.

Toxicity profiles

A total of 104 treatment cycles were given, with a median of 4 cycles (range 1-10) per patient. The main hematological toxicities observed were leucopenia and neutropenia (Table 2). CPT-11 could be escalated up to 300 mg/m^2 without occurence of DLT in the first cycle. One patient treated at the dose level of CPT-11 300 mg/m^2 and R115777 200 mg bid developed grade 4 leuco- and neutropenia complicated by fever in the second cycle, and was taken off study because of progressive disease.

Table 2.

Hematologic toxicity

Level	Cycle I												Cycle II											
*	L				N				T				L				N				Tu			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
A	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
B	-	1	1	-	1	1	-	-	-	-	-	-	-	1	-	1	-	-	1	1	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-	-	-	-	-
D	2	-	-	1	1	-	-	1	-	-	-	1	1	2	-	1	1	1	-	1	-	-	-	-
E	-	-	1	1	-	-	1	1	-	-	2	-	1	-	1	-	1	-	-	1	-	1	-	-
F	-	1	2	-	-	1	2	-	-	-	-	-	1	2	2	-	2	1	-	2	1	-	-	-

* L = Leucopenia, N = Neutropenia, Tr = Thrombocytopenia

At the CPT-11 350 mg/m^2 with R115777 200 mg bid continuous dose level, one patient developed a grade 4 leuco-, neutro- and thrombocytopenia in the first cycle, qualifying for DLT. Remarkably a 4.5-fold higher AUC of R115777 compared to previously treated patients at this dose level was found in this patient, which could have been explained by an overdose of R115777. The patient however denied having taken all the R115777 at once, but (although he stopped early in the cycle) could not return the remaining tablets to us. The question remained unresolved. Having observed DLT, the dose level was expanded with three patients and no further DLT was observed. However continued dosing in subsequent cycles seemed impossible with this schedule, since in three patients treated with continuous R115777 at this level, subsequent doses of CPT-11 had to be reduced because of delayed recovery from hematological toxicity.

The next dose level to be studied was CPT-11 350 mg/m^2 with R115777 200 mg bid intermittently administered. One patient experienced DLT by grade 4 leuco-neutropenia in the first cycle, complicated by an E. Coli sepsis. The patient continued on single agent CPT-11. Three additional patients were treated at this dose level without the occurrence of DLT.

At the dose level studying CPT-11 350 mg/m^2 in combination with R115777 300 mg bid intermittent, hematological toxicity never exceeded grade 3 in the first cycle in the first three patients. Given the fact that CPT-11 was administered at its full single agent dose15 in combination with what seemed to be the recommended single agent dose of R115777 if administered continuously, it was decided to expand this cohort to six patients. Only one out of six patient developed a grade 4 neutropenia in the second cycle, whereas all the other cycles in this cohort (22 cycles) but two could be administered at full dose intensity. Since no MTD was established further escalation of R115777 was performed according the protocol, and is currently ongoing.

Table 3.
Non hematologic toxicity, worst per patient, all cycles

Level	Nausea				Vomiting				Diarrhea				Fatigue			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
A	2		1					1	1				1			1
B	1	2	1		2		1		2	1				1	1	
C		2			1	1			1	1				1		1
D	1	2	1		1	2	1			1	2			1	2	
E	2	2	1		2	1	1		2	1	1			2		
F	3	3			1	3	2		2	2	1		1			

Overall, leuco- and neutropenia was observed more frequent in the second cycle compared to the first cycle. Thrombocytopenia exceeding grade 2 was only observed coinciding with neutropenic fever (Table 2). No platelet transfusions were necessary.

The main non hematological toxicity (Table 3) observed was fatigue. Especially in the continuous R115777 schedules cumulative fatigue was observed in 7 out of 17 patients. One patient was taken off study because of grade 3 fatigue in the second cycle, and returned the normal activity in approximately three weeks after stopping R115777, after which CPT-11 mono-therapy was restarted. This patient did not develop the severe fatigue while on single agent therapy, and experienced a partial response. In another patient who developed grade 3 fatigue R115777 was also interrupted, and after cessation of this toxicity single agent CPT-11 was started. However, this patient developed the same fatigue with single agent CPT-11 as with combination therapy. Both patients did not show deviant pharmacokinetics. It is of particular interest that in patients receiving R115777 in the intermittent schedule, fatigue never exceeded grade 2.

Other types of non-hematological side effects (nausea, vomiting, diarrhea and alopecia) were not severe (Table 3) and in line with what can be expected of single agent CPT-1116. The administration of R115777 did not influence the incidence or severity of these side effects. In all the combination therapy was tolerated remarkably well.

Antitumor activity

Two confirmed partial responses were observed (papilla Vateri carcinoma 27 weeks, ACUP 12 weeks), and another seven patients experienced a prolonged stabilization of their disease (range 12-30 weeks).

Plasma disposition

The plasma concentration-time profiles of each compound (CPT-11, SN-38 and R115777) were similar for all patients studied, with representative examples shown in Figure 1. The pharmacokinetics of unchanged CPT-11 could be best described with a three-compartment model. The kinetic parameters obtained by means of this model are presented in Table 4. Over the various dose ranges studied, the AUC and the peak plasma concentrations of CPT-11 increased in proportion with dose, indicating a linear and dose-independent kinetic behavior. In addition, substantial interpatient variability in parameters was observed (>2-fold), consistent with previous findings, although mean values were strongly correlated to dose, and no significant differences in dose-normalized parameters were observed between the CPT-11 dose levels ($p=0.4268$). Similarly, R115777 dose had no effect on CPT-11 clearance during the first ($p=0.2938$) or second cycle ($p=0.9658$).

In the absence of R115777 (in the first cycle), CPT-11 and SN-38 pharmacokinetics were very similar to previously reported single agent data, with a mean clearance of the parent drug

of 18.5±4.59 L/h/m2 and a moderate degree of interpatient variability of 24.8%, and a mean REC value of 1.73±0.796% [34].

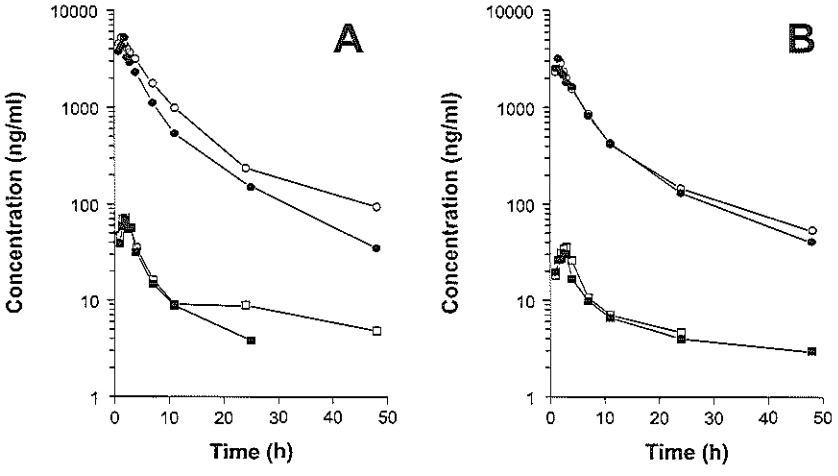


Figure 1. *Plasma concentration versus time profiles of CPT-11 and SN-38 from 2 representative patients treated with CPT-11 at an i.v. dose of 350 mg/m² as a 90-min infusion either given alone (cycle 1) or in combination with a 300-mg bid oral dose of R115777 (cycle 2) given on a continuous (A) or intermittent (B) dosing regimen. CPT-11 (circles) and SN-38 (squares); data from course 1 are indicated by closed symbols and data from course 2 by open symbols.*

In the group of patients treated with the combination with R115777 administered on a continuous schedule, CPT-11 clearance in the second cycle was slightly reduced as compared to the control (p=0.0335). The REC value was unchanged in this group (p=0.1155), whereas the AUC ratio of SN-38 was increased by almost 40% (p=0.0211), suggesting that the increased exposure to SN-38 in the presence of continuous dosing of R115777 was related to an alteration of CPT-11 disposition and not on SN-38 formation or elimination rate. After changing R115777 administration to an intermittent schedule, this kinetic alteration was no longer observed, and both CPT-11 clearance (p=0.0976) as well as the AUC ratio of SN-38 (p=0.0758) were not significantly different between treatment cycles (Table 4).

Table 4.

Summary of CPT-11 pharmacokinetics (mean±S.D.)

Parameter	Day 1	Day 21	difference*	95%C.L.	p
continuous (n=14)					
CL (L/h/m^2)	17.6±4.42	15.4±3.73	2.14±0.901	0.196/4.09	0.0335
MRT (h)	8.59±1.91	10.4±3.91	-1.82±1.18	-4.37/0.731	0.1474
Vss (L/m^2)	149±42.9	158±67.9	-9.64±15.6	-43.3/24.0	0.5465
$T_{1/2}$ (h)	16.7±13.7	16.3±10.5	0.396±2.24	-4.45/5.24	0.8627
AUC_{CPT-11} ratio	1.14±0.200				0.0198
AUC_{SN-38} ratio	1.39±0.412				0.0211
REC (%)	1.92±0.975	2.19±1.04	0.268±0.159	-0.611/0.075	0.1155
intermittent (n=12)					
CL (L/h/m^2)	19.7±4.70	17.8±4.25	1.83±1.01	-0.392/4.05	0.0976
MRT (h)	8.78±2.37	8.79±1.71	-0.018±0.64	-1.42/1.38	0.9785
Vss (L/m^2)	175±81.7	155±42.1	20.5±16.55	-15.9/56.9	0.2412
$T_{1/2}$ (h)	15.4±7.85	12.7±2.22	2.77±2.06	-1.75/7.30	0.2051
AUC_{CPT-11} ratio	1.12±0.221				0.1898
AUC_{SN-38} ratio	1.22±0.345				0.0758
REC (%)	1.51±0.468	1.69±0.743	-0.178±0.138	-0.481/0.125	0.2222

Abbreviations: CL, total plasma clearance; MRT, mean residence time; Vss, volume of distribution at steady-state; $T_{1/2}$, terminal disposition half-life; AUC, area under the plasma concentration versus time curve; REC, relative extent of conversion; 95%C.L., 95% confidence limits for the mean difference; p, probability value from a paired two-tailed Student's t-test

* mean difference with standard error.

The pharmacokinetic behavior of R115777 was also very similar to previously reported single agent data [12] (Table 5). In all patients with two pharmacokinetically evaluable treatment courses (n=15), the mean peak plasma concentration and AUC ratios between the courses were 1.05±0.73 (p=0.4977) and 1.02±0.58 (p=0.9960), respectively. This suggests that CPT-11 does not significantly influence the systemic disposition of the FTI.

Table 5.

Summary of R115777 pharmacokinetics (mean±S.D.)

Parameter	Day 1, cycle 2	Day 8, cycle 1	Ratio*
*200 mg bid group (n=16)***			
C_0 (ng/ml)	98.0±112.1	58.3±51.6	1.25±0.72
T_{max} (h)	2.19±1.05	2.06±0.77	1.12±0.69
C_{max} (ng/ml)	988±805	725±330	1.05±0.73
$AUC_{12\,h}$ (ng.h/ml)	4396±4332	3168±1785	1.02±0.58
300 mg bid group (n=7)			
C_0 (ng/ml)	NA	139±176	NA
T_{max} (h)	2.71±1.25	2.43±0.535	1.17±0.67
C_{max} (ng/ml)	869±467	758±327	1.11±0.30
$AUC_{12\,h}$ (ng.h/ml)	4505±2753	3890±1481	1.09±0.30

Abbreviations: C_0, trough concentration; T_{max}, time to peak concentration; C_{max}, peak plasma concen-tration; AUC, area under the plasma concentration versus time curve; NA, not available due to missing data in 6 patients due to HPLC assay sensitivity limitations (<1.0 ng/ml). * ratio is d8 / d1; ** combined data from patients treated with either the continuous or intermittant schedule.

Discussion

This phase I study was performed to assess the safety and toxicity profile, and to determine the MTD of a combination of the farnesyltransferase inhibitor R115777 and CPT-11. In addition potential pharmacokinetic interaction between the two compounds was investigated. In this interim analysis DLT has not yet been reached, and consequently MTD has not yet been defined.

This study started at continuous dosing of 300 mg R115777 bid with 200 mg/m^2 CPT-11. Based on toxicity from the first patient only it was decided to taper the dose of R115777 to 200 mg bid. Subsequently escalation of CPT-11 in steps of 50 mg/m^2 was performed according to the protocol, and toxicity was only mild. Even at CPT-11 recommended single agent dose, 350 mg/m^2, the schedule was tolerated quite well. We did however observe that 7 out of 17 patients developed cumulative fatigue in this group of patients that were treated with continuous dosing of R115777. Fatigue was also reported in other studies with single agent R115777 [12-14], but is also observed in patients being treated with single agent CPT-11 [15]. Of interest in this is the observation made in the two patients that developed fatigue and

discontinued R115777. After cessation of fatigue, both were treated with single agent CPT-11, and while one again experienced the same fatigue the other experienced none. In a simultaneous combination study with continuous dosing of R115777 however, cumulative fatigue was also observed [35]. This, in combination with the pharmacokinetic observation discussed later, prompted us to change the dosing schedule of R115777 from continuous to intermittent, consisting of R115777 dosing in 14 out of 21 consecutive days.

Formally the change from continuous to intermittent R115777 dosing was a reduction of the total amount of administered medication, and consequently the dose of R115777 ought to be escalated according to the protocol. However, by protocol amendment, first three patients were treated at this CPT-11 350 mg/m^2 with R115777 200 mg bid, but now intermittent, which also gave the opportunity to compare pharmacokinetics between both the continuous and intermittent group of patients at the same dose of both drugs. One of these patients developed DLT, and the dose level was expanded to six patients. Of interest is that no cumulative toxicity was observed at this dose level of the intermittent dosed group, and no cycle needed to be postponed. This in contrast to the patients that were treated with continuous R115777 with the same daily dose, in which three cycles had to be postponed and reduced. This could in theory be explained by the altered metabolism of CPT-11 as discussed later. Moreover, fatigue never exceeded grade 2 in the whole group of intermittent dosed R115777, in contrast to the grade 3 and 4 fatigue observed in the continuous dosed group.

It is of importance to the interpretation of the pharmacokinetics, to consider the dosage schedules. All the patients received CPT-11 on day one in every cycle. All the patients started with R115777 at day three in the first cycle, but at day one in the second and following cycles. In the intermittent dosed group of patients however, R115777 was discontinued for one week before start of a new cycle, in contrast to the continuously dosed patients. Pharmacokinetics were performed during the first two cycles, and therefore the influence from R115777 on the metabolism of CPT-11 could be investigated, and vice versa the influence from CPT-11 on the kinetics of R115777. The metabolism of CPT-11, expressed as the ratio of the AUC of CPT-11 in the cycle combined with R115777 (second cycle) divided by the AUC of CPT-11 in the cycle without R115777 (first cycle), was unaltered in both the continuous and intermittent dosing group (Table 4). The active metabolite SN-38 on the other hand was significantly increased in the continuous R115777 dosing group. R115777, inhibits CYP3A4 in vitro [36]. Unlike for the continuous dosing group, administration of CPT-11 in the intermittent dosing group is preceded in the second cycle by a 1 week wash-out from R115777. Therefore, it is possible that prolonged exposure to R115777 is needed to evoke a significant pharmacokinetic interaction. CYP3A4 inhibitors influence the metabolism of CPT-11 by shifting the routes of metabolism to the carboxyl-esterase pathway, which ultimately leads to a decrease in the inactive metabolites APC and NPC in the plasma, and a increase of plasma SN-38 [37] (Table 3). Although in a rodent model chronic dosing of high concentrations of R115777 did not inhibit diagnostic enzymatic activities [38], it is well

known that animal models are not always representative for human metabolism. For instance, in an in vitro model, conversion of APC to SN-38 by rabbit liver CE has been described [39]. However, rabbit liver CE, though very similar to human liver CE with respect to amino acid sequence, is ~100-fold more efficient in activating APC than the human enzyme in vitro [40]. Indeed, in vitro conversion of APC to SN-38 by human CE or human liver microsomes could not be demonstrated [30]. This could explain the difference in human and animal model pharmacokinetics, and the pharmacokinetic interaction found in this study. Although some authors consider the plasma SN-38 as important considering anti-tumor activity, others stress that only intratumoral carboxyl esterase activity is of importance to the clinical efficacy [21,23,41]. This would consequently mean that only the plasma concentration of CPT-11 is of importance, which in unaltered by the administration of R115777.

We did however observe a significant change in AUC SN-38 between the first and the second cycle in the continuous treated patients, while at intermittent dosing with the same dosage of both drugs, the AUC SN-38 did not change. If we compare continuous to intermittent at the dose level of CPT-11 350 mg/m^2 combined with R115777 200 mg bid, we observed enhanced clinical toxicity at the continuous dose level. Given the small number of patients the clinical relevance of this observation remains uncertain. Still, since the pharmacokinetic alterations match with the clinical observation, we recommend to combine these drugs in an intermittent schedule. Moreover, R115777 in combination therapy is frequently administered intermittently, although pharmacokinetic interaction has not been described before [35,42-45].

At the R115777 300mg bid intermittent dose level, six patients were treated without DLT. In two patients cycles needed to be delayed. One of these patient showed early PD, while the other patient after one delayed cycle received another three cycles without further delay. Overall this schedule was well tolerated. Compared to both studies with single agent CPT-11 and single agent R115777, toxicity of this combination therapy seemed not enhanced. Given the fact that this report is an interim analysis, definite conclusions can not be stated. It can be concluded from these data though, that CPT-11 at its full single agent dose, 350 mg/m^2, in combination with either continuous R115777 200 mg bid or intermittent R115777 300 mg bid is feasible. Given the clinical cumulative toxicity and the pharmacokinetic interaction observed between CPT-11 and continuously administered R115777, in combination with the ongoing discussion on the relevance of plasma SN-38 to antitumor activity, we clearly recommend to prescribe CPT-11 and R115777 intermittently in the 14 out of 21 consecutive days schedule. Since CPT-11 and R115777 have prominent different modes of action, and are clinically active, it is worth studying this combination regimen in phase II/III studies once we have defined MTD.

REFERENCES

1. Ito T, May WS. Drug development train gathering steam. Nat Med. 1996;2:403-404.

2. McCubrey JA, May WS, Duronio V. Serine/threonine phosphorylation in cytokine signal transduction. Leukemia. 2000;14:9-21.
3. Rebollo A, Martinez-A C. Ras proteins: recent advances and new functions. Blood. 1999;94:2971-2980.
4. Gelb MH. Protein prenylation, et cetera: signal transduction in two dimensions. Science. 1997;27:1750-1751.
5. Khosvari-Far R, Cox AD, Kato K. Protein prenylation: key to ras function and cancer intervention. Cell Growth Diff. 1992;3:461-469.
6. End DW. Farnesyl protein transferase inhibitors and other therapies targeting the Ras signal transduction pathway. Invest New Drugs. 1999;17:241-258.
7. Rowinsky EK, Windle JJ, Von Hoff DD. Ras protein farnesyltransferase: a strategic target for anticancer therapeutic development. J Clin Oncol. 1999;17:3631-3652.
8. Du W, Lebowitz PF, Prendergast GC. Cell growth inhibition by farnesyltransferase inhibitors is mediated by a gain of granylgeranylated Rho B. Mol Cell Biol. 1999;19:1831-1840.
9. Reuter CWM, Morgan MA, Bergmann L. Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematologic malignancies. Blood. 2000;96:1655-1669.
10. Lebowitz PF, Prendergast GC. Non-Ras tragets of farnesyltransferase inhibitors: focus on Rho. Oncogene.1998;17:1439-1445.
11. End DW, Smets G, Todd AV. Characterization of the Antitumor Effects of the Selective Farnesyl Protein Transferase Inhibitor R115777 in Vivo and in Vitro, Cancer Res. 2001;61:131-137.
12. Zujewski J, Horak ID, Bol CJ, et al. Phase I and pharmacokinetic study of farnesyl protein transferase inhibitor R115777 in advanced cancer. J Clin Oncol. 2000;18:927-941.
13. Punt CJA, van Maanen L, Bol CJJG. Phase I and pharmacokinetic study of the orally administered farnesyl transferase inhibitor R115777 in patients with advanced solid tumors. Anticancer Drugs. 2001;12:193-197.
14. Karp JE, Lancet JE, Kaufmann SH. Clinical and biologic activity of the farnesyltransferase inhibitor R115777 in adults with refractory and relapsed acute leukemias: a phase 1 clinical-laboratory correlative trial. Blood. 2001;97:3361-3369.
15. Cunningham D, Pyrhonen S, James RD. Randomized trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. Lancet. 1998;352:1413-1418.
16. Rougier P, Van Cutsem E, Bajetta E. Randomised trial of irinotecan versus fluorouracil by continuous infusion after fluorouracil failure in patients with metastatic colorectal cancer. Lancet. 1998;52:1407-1412.
17. Kawato Y, Aonuma M, Hirota Y. Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer Res. 1991;51:4187-4191.
18. Tsuji T, Kaneda N, Kado N. CPT-11 converting enzyme from rat serum: purification and some properties. J Pharmacobio-Dyn. 1991;14:341-349.
19. Rivory LP, Bowles MR Robert J. Conversion of irinotecan (CPT-11) to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), by human liver carboxylesterase. Biochem Pharmacol. 1996;52:1103-1111.
20. Zamboni WC, Houghton PJ, Thompson J. Altered irinotecan and SN-38 disposition after intravenous and oral administration of irinotecan in mice bearing human neuroblastoma xenografts. Clin Cancer Res. 1998;4:455-462.
21. Danks MK, Morton CL, Pawlik CA. Overexpression of a rabbit liver carboxylesterase sensitizes human tumor cells to CPT-11. Cancer Res. 1998;58:20-22.
22. Atsumi R, Okazaki O, Hakusui H. Metabolism of irinotecan to SN-38 in a tumor-isolated tumor model. Biol Pharm Bull. 1995;18:1024-1026.

23. Guichard S, Terret C, Hennebelle I. CPT-11 converting carboxylesterase and topisomerase I activities in tumour and normal colon and liver tissues, Br J Cancer 1999;80:364-370.
24. Iyer L, King CD, Whitington PF. Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. J Clin Invest. 1998;101:847-854.
25. Ciotti M, Basu N, Brangi M. Glucuronidation of 7-ethyl-10-hydroxycampto-thecin (SN-38) by the human UDP-glucuronosyltransferases encoded at the UGT1 locus. Biochem Biophys Res Comm. 1999;260:199-202.
26. Rivory LP, and Robert J. Identification and kinetics of a β-glucuronide metabolite of SN-38 in human plasma after administration of the camptothecine derivative irinotecan. Cancer Chemother Pharmacol. 1995;36:176-179.
27. Haaz MC, Rivory LP, Jantet S. Glucuronidation of SN-38, the active metabolite of irinotecan, by human hepatic microsomes. Pharmacol Toxicol. 1997;80:91-95.
28. Lokiec F, Monegier du Sorbier B. Irinotecan (CPT-11) metabolites in human bile and urine. Clin Cancer Res. 1996;2:1943-1949.
29. Haaz MC, Rivory LP, Riché C. Metabolism of irinotecan (CPT-11) by human hepatic microsomes: participation of cytochrome P-450 3A and drug interactions. Cancer Res. 1998;58:468-472.
30. Rivory LP, Riou JF, Haaz MC. Identification and properties of a major plasma metabolite of irinotecan (CPT-11) isolated from the plasma of patients. Cancer Res. 1996;56:3689-3694.
31. Dodds HM, Haaz MC, Riou JF. Identification of a new metabolite of CPT-11 (irinotecan). Pharmacological properties and activation to SN-38. J Pharmacol Exp Ther. 1998;281:578-583.
32. De Bruijn P, De Jonge MJA, Verweij J. Femtomole quantitation of 7-ethyl-10-hydroxycamptothecine (SN-38) in plasma samples by reversed-phase high-performance liquid chromatography. Anal Biochem. 1999;269:174-178.
33. Sparreboom A, De Jonge MJA, De Bruijn P. Irinotecan (CPT-11) metabolism and disposition in cancer patients. Clin Cancer Res. 1998;4:2747-2754.
34. Kehrer DFS, Soepenberg O, Loos WJ. Modulation of camptothecin analogs in the treatment of cancer: a review. Anti-Cancer Drugs. 2001;12:89-105.
35. Piccart-Gebhart M, Branle F, de Valeriola D. A phase I, clinical and pharmacokokinetic trial of the farnesyl transferase inhibitor R115777 + docetaxel: a promising combination in patients with solid tumors. Pro Am Soc Clin Oncol 2001; 20: abstract 318.
36. Bohets H. An in vitro study on the microsomal cytochrome P-450 form(s) involved in the metabolism of ^{14}C-R115777 and on the effect of R115777 on the metabolism of specific human cytochrome P-450 substrates. Janssen Research Foundation, Non-clinical Pharmacokinetics Report R115777/FK2809. June 1998.
37. Kehrer DFS, Verweij J, de Bruijn P. Modulation of CPT-11 metabolism in cancer patients by ketoconazole . Proc Am Assoc Cancer Res. 2001; abstract 2897.
38. Mannens G. Study on the possible induction and/or inhibition of hepatic drug metabolizing enzymes by R115777 in male and female SPF Wistar rats, after oral administration by gavage for three months. Janssen Research Foundation. Non-clinical Pharmacokinetics Report R115777/FK2795. June 1998.
39. Guichard SM, Morton L, Krull EJ. Conversion of the CPT-11 metabolite APC to SN-38 by rabbit carboxylesterase. Clin Cancer Res. 1998;4:3089-3094.
40. Danks MK, Morton CL, Krull EJ. Comparison of the efficiency of CPT-11 activation by a rabbit and a human carboxylesterase for use in enzyme/prodrug therapy. Proc Am Assoc Cancer Res. 1999;40: abstract 731.
41. Ratain MJ, Innocenti F, Vogelzang NJ. Modulation of irinotecan (CPT-11) toxicity and pharmacokinetics by cyclosporine and phenobarbital. Proc Am Soc Clin Oncol 2001;20: abstract 74a .

42. Holden S, Eckhardt S, Fischer S. A phase I pharmacokokinetic and biological study of the farnesyl transferase inhibitor R115777 and capecitabine in patients with advanced solid malignancies. Proc Am Soc Clin Oncol 2001;20: abstract 316.
43. Liebes L, Hochster H, Speyer J. Enhanced myelosuppression of topotecan when combined with the farnesyl transferase inhibitor R115777: a phase I and pharmacodynamic study. Proc Am Soc Clin Oncol 2001;20: abstract 321.
44. Adjei A, Erlichman C, Marks R. A phase I trial of the farnesyl transferase inhibitor R115777, in combination with gemcitabine and cisplatin in patients with advanced cancer. Proc Am Soc Clin Oncol 2001;20: abstract 320.
45. Schwartz G, Rowinsky E, Rha S. A phase I pharmacokokinetic and biological correlative study of R115777 and trastuzumab in patients with advanced cancer. Proc Am Soc Clin Oncol 2001;20: abstract 322.

Summary, Conclusions and Future Perspectives

This thesis includes phase I and pharmacological studies on topoisomerase I inhibitors and/or in combination with a farnesyl transferase inhibitor, aminoglycoside antibiotic and CYP3A4 inhibitor.

Topoisomerase I inhibitors are an important relatively new class of anti cancer drugs, with a unique mode of action. Topoisomerase I is a nuclear enzyme involved in the replication of DNA, by forming a covalent binding with DNA, the cleavable complex. Topoisomerase I inhibitors bind to the cleavable complex, resulting in a single strand DNA break, which ultimately leads to cell death.

In **Chapter 1** an overview of the current topoisomerase I inhibitors and possible ways to modulate these, are summarized. The topoisomerase I inhibitors reviewed in this paper are all semisynthetic analogues of camptothecin. We present preclinical and clinical data on camptothecin analogues that are already being used in clinical practice [i.e., topotecan and irinotecan (CPT-11)] or are currently in clinical development (e.g., 9-aminocamptothecin, 9-nitrocamptotecin, lurtotecan, DX 8951f and BN 80915), as well as drugs that are still only developed in a preclinical setting (Silatecans, polymer-bound derivates) . A variety of different strategies is being used to modulate the systemic delivery of this class of agents, frequently in order to increase antitumor activity and/or reduce experienced side effects. Three principal approaches are being discussed, including (i) pharmaceutical modulation of formulation vehicles, structural alterations and the search for more water soluble prodrugs, (ii) modulation of routes of administration and considerations on infusion duration, and (iii) both pharmacodynamic and pharmacokinetic biomodulation.

In **Chapter 2** a method to determine liposomal lurtotecan (NX211) in human plasma and urine is described. In the context of the following study, we developed sensitive RP-HPLC methods with fluorescence detection using a sample clean-up procedure that disrupts the liposomes, thus enabling determination of total drug levels in plasma and urine samples following NX 211 administration. The methods have been validated in terms of sensitivity, accuracy and precision, and have been used in a pharmacokinetic experiment in a patient to investigate their applicability *in vivo*.

Chapter 3 reports on the phase I open-label, dose-escalating trial that was initiated to determine the maximum-tolerated and recommended dose, toxicity profile and pharmacokinetics of the liposomal topoisomerase I inhibitor, lurtotecan (NX 211) administered as a 30-minute IV infusion once every 3 weeks in cancer patients. Dose escalation decisions were based upon all toxicities during the first cycle as well as pharmacokinetic parameters. Serial plasma, whole blood, and urine samples were collected for up to 96 hours following the end of infusion and drug levels were determined by high-performance liquid chromatography as described in the earlier chapter.

Twenty-nine patients received 77 courses of NX 211 at six different dose levels ranging from 0.4 to 4.3 mg/m^2. Neutropenia and thrombocytopenia were the dose-limiting toxicities, and were not cumulative. Other toxicities, including nausea, vomiting and fatigue, were mild to moderate. Nine patients had stable disease while on treatment, whereas one of these with an adenocarcinoma of unknown origin (ACUP) had a significant regression just not reaching the criteria of partial response (PR). The systemic clearance of lurtotecan in plasma and whole blood was 0.82±0.78 and 1.15±0.96 L/h/m^2, respectively. Urinary recovery (Fu) of lurtotecan was 10.1±4.05% (range, 4.9 to 18.9). In contrast to systemic exposure measures, the dose excreted in urine was significantly related to the percent decrease in neutrophil and platelet count at nadir (P<.00001). The recommended dose for phase II studies is 3.8 mg/m^2 once every three weeks. Pharmacologic data suggest a relationship between exposure to lurtotecan and NX 211-induced clinical effects.

In **Chapter 4** the additional chromatographic peak observed in plasma samples of patients receiving NX 211 is described. We have isolated and purified this product by sequential solid-phase extractions, and report its structure and cytotoxicity relative to lurtotecan and related agents. NMR data indicate that cleavage of the piperazino moiety occurred at the N-C bond of the B-ring, yielding 7-methyl-10,11-ethylenedioxy-20(*S*)-camptothecin (MEC). Tests of the growth inhibition potential of MEC in 7 human tumor cell lines showed that the compound was approximately 2 to 18-fold more cytotoxic than lurtotecan, topotecan and SN-38. Subsequently, we found that MEC was the product of rapid photolysis of lurtotecan, with the rate of degradation inversely proportional to NX 211 concentrations, and greatly depend on light intensity. Furthermore, MEC concentrations were found to significantly increase in plasma samples exposed to laboratory light, but not in blood. MEC was not produced from NX 211 in the presence of human liver microsomes, suggesting that is not a product of CYP P450 metabolism. Using a validated analytical method, trace levels of MEC were quantitated in blood samples of 2 patients. These observations confirm that the precautions for protection from light currently specified for preparation and administration of NX 211 dose solution are critical. Procedures to minimize formation of MEC, by the use of amber vials for NX 211 and by preparation of dilutions immediately before clinical use in a fashion totally protected from light, are now being routinely implemented.

The high specificity in the mechanism of action of topoisomerase I inhibitors for the S-phase in the cell cycle has led to the recognition that the compounds may require prolonged exposure to maximize the fractional cell kill. In this light we investigated extended metabolism of CPT-11 in **Chapter 5**. The active metabolite of CPT-11, SN-38, is either formed through enzymatic cleavage of CPT-11 by carboxyl esterases (CE) or through cytochrome P450 3A-mediated oxidation to NPC, and a subsequent conversion by CE. In the

liver, SN-38 is glucuronidated (SN-38G) by UGT1A1, which also conjugates bilirubin. Fourteen patients that were treated with 350 mg/m^2 CPT-11 are described, and pharmacokinetic analysis was performed during a 500-h collection period. The half-life and area under the plasma concentration-time curve of SN-38 were found to be a 2-fold higher as compared to earlier reported estimates. As an explanation for this phenomenon, we noted substantial formation of SN-38 from CPT-11 and NPC by plasma CE, consistent with the low circulating levels of NPC observed. In addition, transport studies in Caco-2 monolayers indicated that non-glucuronidated SN-38 could cross the membrane from apical to basolateral, indicating the potential for re-circulation processes that can prolong circulation times. Interestingly, individual levels of fecal β-glucuronidase, which is known to mediate SN-38G hydrolysis, were not related to any of the SN-38 kinetic parameters, suggesting that this enzyme does not play a role in SN-38 re-circulation. We have also found, in contrast to earlier data, that SN-38G/SN-38 plasma concentration ratios decrease over time from ~7 (up to 50 h) to ~1 (at 500 h). This decrease could be explained by the fact that glucuronidation of SN-38 and bilirubin is increasingly competitive at lower drug levels. In addition, no evidence was found for SN-38G transport through the Caco-2 cells. These findings indicate that until now the circulation time of SN-38 has been underestimated. This is of crucial importance to our understanding of the clinical action of CPT-11 and for future pharmacokinetic/pharmaco-dynamic relationships.

Irinotecan (CPT-11) induced diarrhea is a frequent and often dose limiting toxicity in the treatment of colon cancer. Recently, it was suggested from animal models that β-glucuronidases produced by microflora in the large bowel may play a major role in the development of CPT-11-induced diarrhea by mediating hydrolysis of SN-38G to form the active SN-38. By eliminating the β-glucuronidases producing bacteria with an antibiotic, this hydrolysis could be blocked. The study described in **Chapter 6** was designed to evaluate CPT-11 disposition and pharmacodynamics in the presence and absence of the broad-spectrum antibiotic neomycin. Seven evaluable cancer patients experiencing diarrhea grade ≥2 after receiving CPT-11 alone, received the same dose combined with oral neomycin at 1000 mg t.i.d. (days –2 to 5) in the second course. Neomycin had no effect on the systemic exposure of CPT-11 and its major metabolites. However, it changed fecal β-glucuronidase activity to undetectable levels, and decreased fecal concentrations of the pharmacologically active metabolite SN-38. Although neomycin had no significant effect on hematological toxicity, diarrhea ameliorated in 6 of 7 patients. Our findings indicate that bacterial β-glucuronidase plays a crucial role in CPT-11-induced diarrhea without affecting enterocycling and systemic SN-38 levels.

Chapter 7 describes a high-performance liquid chromatographic assay with UV detection that has been developed for the determination of ketoconazole in human plasma. Quantitative extraction was achieved by a single solvent extraction involving a mixture of acetonitrile-*n*-butyl chloride. Ketoconazole and the internal standard (clotrimazole) were separated on a column packed with Inertsil ODS-80A material and a mobile phase composed of water-acetonitrile-tetra-hydrofuran-ammonium hydroxide-triethylamine. The column effluent was monitored at a wavelength of 206 nm with a detector range set at 0.5. The calibration graph was linear in the range of 20-2000 ng/ml, with a lower limit of quantitation of 20.0 ng/ml. The extraction recoveries for ketoconazole and clotrimazole in human plasma were 93±9.7% and 83±10.0%, respectively. The developed method has been successfully applied to a clinical study to examine the pharmacokinetics of ketoconazole in a cancer patient.

There is considerable motivation for understanding adverse drug interactions with anticancer agents because of their narrow therapeutic index and the numerous concomitant medications that are administered routinely or intermittently. Indeed, drug interactions with anticancer agents are a major cause of morbidity and mortality in modern medical practice. The consequences of simultaneous treatment of CPT-11 with a potent enzyme inhibitor, ketoconazole, in a group of cancer patients is described in **Chapter 8**. A total of 7 evaluable patients was treated in a randomized cross-over design with CPT-11 (350 mg/m^2) given alone, followed 3 weeks later by CPT-11 (100 mg/m^2) in combination with ketoconazole, or vice versa. Serial plasma, urine and feces samples were obtained and analyzed for CPT-11 and the metabolites SN-38, SN-38-G, NPC and APC. With ketoconazole co-administration, the formation of APC was reduced by 87%, while the relative exposure to the CE-mediated SN-38 was increased by 110%. These metabolic alterations occurred without substantial changes in CPT-11 clearance and formation of SN-38G. In spite of the reduced dose in the combination course, no significant differences in toxicity profiles were noted. Parallel *in vitro* studies indicated that ketoconazole had no influence on circulating CE activity, or on CYP3A4-mediated SN-38 metabolism in human liver microsomes. Overall, inhibition of CYP3A4 in cancer patients leads to significantly increased formation of SN-38 following CPT-11 administration. Simultaneous administration of various commonly prescribed drugs (either substrate or inhibitor of CYP3A4) can potentially result in fatal outcomes, and up to 4-fold reductions in CPT-11 dose are indicated.

Given the distinctly different mode of action of R115777 and CPT-11, and the need for more efficacious treatment of (colorectal) cancer, a strong rationale exists for investigating a CPT-11 based regimen in combination with R115777. In **Chapter 9** we present a preliminary analysis of the clinical observations and pharmacokinetics of R115777, CPT-11 and its metabolites, in patients who were treated in a phase I dose finding study with oral R115777

bid, in combination with intravenous CPT-11, given once every three weeks. At the first dose level R115777 was administered continuously, but starting at day 3 in the first cycle, enabling pharmacokinetic interaction assessment. In the second part of the study intermittent administration of R115777 was assessed. The dose of both drugs was escalated, for CPT-11 up to the recommended single agent dose, 350 mg/m^2 once every three weeks, and for R115777 up to dose limiting toxicity. Pharmacokinetics were performed during the first two cycles. Twenty-nine patients with a median age of 52 years (range 34-75), median ECOG-PS 1 (range, 0-1), received 104 cycles. The combination was tolerated reasonably well up to the level of CPT-11 350 mg/m^2 with continuous R115777 200 mg bid. The toxicities most frequently observed were leucopenia and neutropenia. The frequency and severity of nausea, vomiting and diarrhea seemed to parallel those of single agent CPT-11. At the continuous R115777 schedule, the area under the curve (AUC) of SN-38 in the second cycle was significantly higher than in the first cycle (p=0.0211), as was the AUC of CPT-11 (p=0.0335). Because of clinical and pharmacokinetic findings, the administration of R115777 was changed from continuous to intermittent dosing in the second part of the study. In contrast to the continuous R115777 dosing, the AUC of SN-38 and CPT-11 did not alter with intermittent R115777 dosing (p=0.0758). Fatigue was more pronounced in the patients treated in the continuous schedule than with the intermittent schedule. Currently, the combination with escalating doses of intermittent R115777 bid is under investigation. Although the MTD has not yet been reached, the combination therapy of CPT-11 at its full single agent dose with either continuous R115777 200 mg bid or intermittent R115777 300 mg bid is feasible. Continuous dosing of R115777 does influence the disposition of CPT-11, although the clinical relevance of this observation has not been resolved.

Topoisomerase I inhibitors are among the most promising new anticancer drugs that have been developed in recent years. Topotecan and CPT-11 are now registered for the treatment of ovarian and colon cancer, respectively. The unique mechanism of action on topoisomerase I and activity against a broad spectrum of other malignancies are an ongoing stimulus for further clinical development. With the growing knowledge on pharmacodynamics and pharmacokinetics of the different topoisomerase I inhibitors, the poor water solubility and pH-dependent reversible interconvention between the active lactone and inactive carboxylate form, as well as increase in activity or stability by substitutions to specific sites on the molecule, much effort has and will be done to increase antitumor activity of this group of drugs. Meanwhile pharmacological modulation, particularly of CPT-11, can be of interest to reduce toxicity and to influence metabolic pathways. In this light we currently investigate the clinical applicability of profylactic treatment of irinotecan induced diarrhea by co-treatment with neomycin, in a double blind randomized multi-center study.

Although much effort is being put into development of new analogues, the question to answer remains if drugs under development will ultimately lead to the theoretically expected

higher activity and/or reduced toxicity. Last but not least optimization of schedules, routes of administration and combination therapies will lead to numerous new studies in different tumor types. It is to be expected that in the future many new formulations and/or combinations will be developed. In contrast to other previously registered anticancer drugs, the pharmacological knowledge on how topoisomerase I inhibitors behave in humans will lead to a more logic and efficient development of these agents.

Samenvatting en Conclusies

Dit proefschrift bevat studies met topoisomerase I remmers, een relatief nieuwe groep antikanker middelen (cytostatica) met een uniek werkingsmechanisme.
Tumoren groeien per definitie snel. Dit komt doordat de cellen waaruit de tumor is opgebouwd een hoge delingssnelheid hebben. Bij elke celdeling verdubbelt het DNA zich ook. Het enzym topoisomerase I speelt een centrale rol bij de DNA replicatie. Door dit enzymatische proces te remmen, met een zogenaamde topoisomerase I remmer, wordt de DNA replicatie geblokkeerd. Hierdoor treedt er een DNA breuk op in de zich delende tumorcel, waardoor deze uiteindelijk te gronde gaat. Inmiddels hebben twee verschillende soorten topoisomerase I remmers een vaste plaats in de behandeling van kanker gekregen. Een hiervan is irinotecan (CPT-11, zie ook hoofdstukken 5, 6, 8 en 9) dat gebruikt wordt voor de behandeling van dikke darm kanker

Alle cytostatica, en dus ook topoisomerase I remmers, hebben naast het gewenste effect op tumorcellen ook ongewenste bijwerkingen op andere lichaamscellen, en wel het meest op snel delende cellen. Een bekend voorbeeld hiervan is kaalheid door haaruitval. Een ander voorbeeld zijn witte bloedcellen in het beenmerg die te gronde gaan, zodat er een tekort aan deze cellen ontstaat. Dit heeft tot gevolg dat de afweer van de patiënt tegen bacteriële infecties, gedurende korte tijd, sterk afneemt.

In **hoofdstuk 1** wordt een overzicht gegeven van de op dit moment bekende topoisomerase I remmers die in klinische ontwikkeling en/of gebruik zijn. Wij beschrijven de farmaceutische modulatie van topoisomerase I remmers, waarbij door veranderingen aan de structuurformule stoffen als CPT-11 en lurtotecan zijn gevormd. Onder farmaceutische modulatie vallen ook de verschillende verpakkingsvormen, zoals bijvoorbeeld lurtotecan dat in een liposoom verpakt is, zoals beschreven in de hoofdstukken 2 tot 4. Vervolgens worden verschillende toedienings routes beschreven zoals een pil, zalf, infuusvloeistof alsmede verschillende toedienings schema's. Als laatste komt farmaco-kinetische en -dynamische biomodulatie aan de orde, ofwel het ingrijpen op de biologische omzetting van topoisomerase I remmers zoals die plaatsvindt in het lichaam, waarvan de hoofdstukken 6 en 8 voorbeelden zijn.

Hoofdstuk 2 beschrijft de methode waarmee het farmaceutisch gemoduleerde liposomaal lurtotecan (NX211) in bloed en urine door ons gemeten wordt. Het is bekend dat langdurige blootstelling aan topoisomerase I remmers de anti-tumor activiteit doet toenemen. De genoemde topoisomerase I remmer zit verpakt in een liposoom, een soort vetdruppeltje, dat per infuus wordt toegediend. Het langzaam uit dit vetdruppeltje wegsiepelende medicijn zou de anti-tumor activiteit gunstig kunnen beïnvloeden

In **hoofdstuk 3** wordt de fase I studie met NX211 beschreven, ter bepaling van de maximaal aan patiënten toedienbare dosering met dit medicijn. Naast de klinische haalbaarheid en anti-tumor activiteit, beschrijven wij ook de farmacokinetiek. Hierbij wordt een unieke correlatie tussen een farmacokinetische bevinding en klinisch relevante toxiciteit

gevonden. Het blijkt dat de hoeveelheid in de urine uitgescheiden lurtotecan een relatie heeft met de daling van het aantal witte bloedcellen. Wij veronderstellen dat de hoeveelheid in de urine uitgescheiden lurtotecan een afspiegeling is van de in de bloedbaan aanwezige hoeveelheid vrij lurtotecan, dat wil zeggen biologisch actief lurtotecan, wat zich buiten de liposomen bevindt en ten dele in de urine wordt uitgescheiden.

Bij de bepalingen van NX211 uit het bloed vonden wij een onbekend stofje. In **hoofdstuk 4** beschrijven wij hoe wij dit stofje hebben geïsoleerd, gepurificeerd en de structuurformule hebben gedefinieerd. Middels proeven met deze niet eerder beschreven stof, MEC, op gekweekte tumorcellen, tonen wij aan dat deze stof uiterst giftig is, een tiental maal giftiger dan lurtotecan. MEC blijkt een fotodegradatie product van lurtotecan te zijn, hetgeen betekent dat MEC ontstaat door NX211 aan (zon)licht bloot te stellen. Ter voorkoming van fotodegradatie bij de toediening van NX211 wordt deze nu onder strikte lichtprotectie gegeven.

Het is bekend dat langdurige blootstelling aan topoisomerase I remmers de anti tumor activiteit verbetert. De topoisomerase I remmer CPT-11 wordt eenmaal per drie weken middels een infuus toegediend. CPT-11 zelf is een niet werkzame stof, maar wordt onder andere in het lichaam door een reactie met carboxylesterase (CE) omgezet tot SN-38, dat een zeer sterke antikanker werking heeft. De omzetting (metabolisme) van CPT-11 is zeer gecompliceerd (zie kaft van dit proefschrift). Om beter inzicht in het metabolisme te krijgen hebben wij bij patiënten die behandeld worden met CPT-11 bloedmetingen verricht tot 500 uur na de toediening hiervan. In **hoofdstuk 5** wordt deze farmacologische studie beschreven, waarbij als meest opvallende bevinding blijkt dat de geschatte hoeveelheid van de circulerende actieve metaboliet SN-38, in realiteit tweemaal meer blijkt te zijn dan voorheen werd gedacht. In dit hoofdstuk ga ik in op een mogelijke verklaring voor deze observatie.

Een ernstige en frequente bijwerking van CPT-11 is de laat ontstane diarree, die bij ongeveer vijftig procent van de patiënten optreedt. Alhoewel er redelijke therapie bestaat om deze diarree weer onder controle te krijgen bij het leeuwendeel van de patiënten, zou een therapie ter voorkoming van de diarree te prefereren zijn. In **hoofdstuk 6** beschrijven wij hoe een, in de ontlasting voorkomende inactieve metaboliet SN-38-G (die in de lever gevormd is uit het actieve SN-38), weer teruggevormd kan worden naar de actieve metaboliet SN-38. Deze reactivatie geschiedt door β-glucuronidase dat in de darmbacterie zit. Door nu deze bacterie met een antibioticum uit te roeien voorkom je vorming van de actieve metaboliet SN-38 in de ontlasting. Hoge concentraties van SN-38 in de darm kunnen locaal schade geven aan de darmwand, met als uiteindelijk gevolg laat optredende diarree. Dit proces zou door antibiotische therapie voorkomen kunnen worden. In deze pilotstudy hebben wij aangetoond dat toediening van het antibioticum geen invloed heeft op het metabolisme van CPT-11 in het bloed, waaruit blijkt dat je dit middel ongestraft gelijktijdig met CPT-11 kan voorschrijven aan een patiënt. Tegelijkertijd tonen wij aan dat de β-glucuronidase activiteit in de ontlasting bijna geheel verdwijnt en er nauwelijks nog SN-38 in de ontlasting aantoonbaar is. Bij 7

patienten die tijdens de eerste kuur met CPT-11 diarree hadden ontwikkeld gaven wij voorafgaand aan de tweede kuur antibiotica. 6 van de 7 patienten hadden geen diarree meer tijdens de tweede kuur.

In **hoofdstuk** 7 beschrijven wij een methode die wij ontwikkeld hebben om ketoconazol concentraties in het bloed te meten. Ketoconazol, dat behalve een frequent aan patiënten voorgeschreven anti-schimmel middel is, is tevens een potente remmer van het CYP3A4 (zie kaft van dit proefschrift). Onder invloed van CYP3A4 wordt CPT-11 omgezet in de niet werkzame metabolieten NPC en APC. De primaire vraag die aan de orde is gesteld is welke invloed ketoconazol op het metabolisme van CPT-11 heeft en hoe belangrijk deze is. In **hoofdstuk 8** beschrijven wij de interactie tussen deze twee middelen, door patiënten achtereenvolgens eerst met CPT-11 alleen te behandelen, en vervolgens met (een 3.5x gereduceerde dosis) CPT-11 en ketoconazol. Om tot een vergelijk te komen werd bij beide kuren op vaste tijdstippen metingen uit het bloed gedaan. Het blijkt dat door remming van het CYP3A4 de hoeveelheid NPC/APC met een factor twee afneemt, terwijl de reactie via CE (zie hoofdstuk 5) met een factor twee toeneemt. Ergo er ontstaat een dubbele dosis van het actieve, maar ook giftige SN-38 in het bloed. Wij concluderen dat gelijktijdige toediening van een volle dosis CPT-11 en een CYP3A4 remmer, kan leiden tot ernstige en zelfs lethale toxiciteit.

In **hoofdstuk 9** beschrijven wij een fase I studie met CPT-11 en de farnesyltransferase remmer R115777. Deze beide tegen kanker effectieve middelen hebben volstrekt andere werkingsmechanismen. Daarmee bestaat er een duidelijke rationale om deze combinatie therapie te onderzoeken. Naast de klinische haalbaarheid en anti-tumor activiteit, beschrijven wij ook de farmacokinetiek. Het blijkt dat continue dosering van het oraal toegediende R115777, het metabolisme van CPT-11 beïnvloedt. Dit heeft, in combinatie met de klinische observatie van cumulatieve vergiftigingsverschijnselen, geleid tot het besluit de R115777 voor te schrijven in een intermitterend schema. Omdat wij in deze studie de maximaal tolereerbare dosering nog niet hebben bereikt, is deze studie nog steeds gaande.

Topoisomerase I remmers behoren tot de meest veelbelovende anti kanker middelen die zijn ontwikkeld in de afgelopen jaren. Het unieke werkingsmechanisme in combinatie met de activiteit tegen een breed spectrum van tumoren zijn een continue stimulans voor verdere klinische ontwikkeling. Door een groeiende kennis over de farmacokinetiek en farmacodyamiek van deze groep van cytostatica, is en zal er veel onderzoek worden verricht teneinde de antitumor effectiviteit verder te verhogen. Tevens is farmacologische modulatie, in het bijzonder van CPT-11, van interesse teneinde toxiciteit te verminderen dan wel te beïnvloeden. In dit licht voeren wij momenteel een dubbelblind gerandomiseerd onderzoek uit om de klinische werkzaamheid van profylactische antibiotica tegen CPT-11 gerelateerde diarree te bewijzen.

Ondanks alle energie die er in de ontwikkeling van de topoisomerase I remmers wordt gestopt is het niet zeker of dit uiteindelijk zal leiden tot theoretisch verwachte effectievere en minder toxische middelen. Daarnaast zullen er nog veel studies verricht worden om de optimale dosering, schema's en/of combinatie-therapieën te onderzoeken. In tegenstelling tot de ontwikkeling van eerder geregistreerde cytostatica, zal de farmacologische kennis over hoe topoisomerase I remmers zich in mensen gedragen, leiden tot een logischer en efficiëntere ontwikkeling van deze middelen.

Dankwoord

Nadenkend over het dankwoord passeren meer mensen mijn gedachten dan ik zal kunnen vernoemen in deze tekst. Allereerst dank ik alle patiënten die geconfronteerd met hun ziekte hun vertrouwen hebben gesteld in de medische wetenschap en belangeloos hebben meegewerkt aan deze onderzoeken in de strijd tegen kanker. Volledig geïnformeerd, levend tussen hoop voor henzelf en voor die van andere patiënten in de toekomst, vormen zij de hoeksteen van dit proefschrift.

Prof. Dr. Verweij, promotor, beste Jaap, jou ben ik veel dank verschuldigd. Ik heb grote bewondering voor de stuurmanskunst waarmee je mijn promotieonderzoek richting hebt gegeven. Naast je gestructureerde aanpak en je altijd vriendelijke doch resolute correcties, vergeet ik nooit hoe je onverwijld ruimte gaf wanneer dat echt nodig was. Wel hoop ik dat jij vergeet hoe ik je meermalen onder tijdsdruk heb gezet. Na vele verhalen gehoord te hebben geloof ik niet dat er een betere promotor in Nederland rondloopt, ik prijs mij gelukkig.

Dr. De Jonge, copromotor, beste Maja, ik zal nooit vergeten hoe je mij groothartig de neomycine-studie overdroeg en jezelf naar de laatste auteursplaats verjoeg. Veel dank heb ik voor de secure en stimulerende hulp die je mij altijd bood bij het opstellen van manuscripten en protocollen. Je verdient meer eer dan je bescheidenheid doet voorkomen.

Dr. Sparreboom, copromotor, beste Alex, dank voor de vriendschappelijkheid waarmee je me in de mij onbekende wereld van de farmacologie, welhaast spelenderwijs, wegwijs hebt gemaakt. Je altijd stimulerende grote enthousiasme was een continue bron van vreugde. Je welgemeende complimenten geven eenieder energie en inspiratie. Jij weet promoveren tot een waar feest te maken.

Prof. Dr. Stoter, beste Gerrit, ik dank je voor het voorrecht dat ik heb gekregen om te mogen promoveren op de door jou zo voortreffelijk opgezette en aangestuurde afdeling oncologie. De manier waarop je problemen weet te deduceren tot de essentie is een bewonderenswaardige eigenschap.

Farmacologie laboranten, Peter, Walter, Desiree en Eric, ik kan mij geen dinsdagmiddag meer zonder jullie voorstellen. Jullie gedrevenheid maakt farmacologisch onderzoek spannend. Dr. Loos, beste Walter, vooral jou wil ik danken voor je enorme inzet en groot analytisch- en doorzettings vermogen. Jij wist van NiX iets spectaculairs gemaakt. Het feit dat het voor jou toch niks werd, heb je ruiterlijk gedragen.

Research verpleegkundigen, Carla, Miranda, Linda, Christel en jullie voorgangsters, zonder jullie zou geen onderzoek kunnen lopen. Dank voor alle praktische tips die protocollen echt uitvoerbaar maken en voor het flexibel inspringen wanneer iemand weer eens een steekje had laten vallen.

Datamanagers, Judith, Gerda, Jacqueline en alle anderen, zonder jullie monnikenwerk zou de berg resultaten van mijn studies een grote nachtmerrie zijn geworden. Heel veel dank voor jullie eindeloze puzzelwerk.

Verpleging en baliemedewerksters, op zowel klinische als poliklinische afdelingen, zonder jullie hartverwarmende inzet zouden patiënten zich verloren voelen en zou onderzoek verloren gaan.

Collegae Junioren, zelden of nooit was ik onderdeel van een zo gezellige en diverse groep dokters. Of het nu in de Daniel of daarbuiten was, ik heb er mateloos van genoten de tijd met jullie te delen. Hans, dank voor je aanbod een mijner paranimfen te zijn, ik aanvaard het met plezier.

Collegae stafleden, dank voor de kennis en kamers die jullie met mij hebben willen delen. Het is een voorrecht om in jullie kliniek tot oncoloog te worden opgeleid.

Secretariaat medewerksters, hoe jullie de organisatie georganiseerd weten te houden is mij een raadsel. Petra, veel dank voor de lay-out van mijn proefschrift.

Familie, in het bijzonder mijn lieve ouders, als ik later groot ben dan word ik.....de liefdevolle jeugd die jullie mij schonken heeft zich dieper vastgezet dan in herinneringen alleen. Daarnaast blijft het heerlijk om dagelijks met jullie van gedachten te kunnen wisselen. Zonder Marieke als morele steunpilaar bij de komende gedachtewisselingen, zou de promotiedag niet compleet zijn.

Lieve Christine, zonder jou was dit proefschrift niet van de grond gekomen. Alle tijd die ik niet kon geven aan de kinderen was jij dubbel voor hen aanwezig. Jou onaflatende inspanning van de laatste drie jaar om iedereen in ons gezin gelukkig te laten zijn, staat niet in verhouding tot de inspanning die tot dit proefschrift heeft geleid.

Curriculum Vitae

Diederik Kehrer is geboren op 17 december 1960 te Voorthuizen. In 1980 ving hij zijn studie geneeskunde aan te Amsterdam. Naast zijn studie had hij het genoegen om het IX-de lustrum van zijn dispuut S.I.R.I.U.S. te organiseren. Na het behalen van zijn doctoraal examen werkte hij, in zijn wachttijd voor aanvang van de co-assitentschappen, als onderzoekscoordinator in het Onze Lieve Vrouwe Gasthuis. In die periode heeft hij tevens een half jaar in het Central Jalma Institute for Leprosy in Agra – India - gewerkt. Na een tijd van Agnio-schap ving hij de opleiding interne geneeskunde in het Onze Lieve Vrouwe Gasthuis (Dr Silberbusch) aan, waarvan de laatste twee jaar in het Academisch Medisch Centrum (prof Dr Briët) werden doorgebracht. Tot op heden is hij werkzaam in het Academisch Ziekenhuis Rotterdam locatie Daniel den Hoed Kliniek (prof Dr Stoter).
In 1992 is hij getrouwd met Christine Kehrer-Bot aan wie hij ondermeer zijn allerliefste Lotte (1993), Nienke (1995) en Hidde (1996) dankt.

Publications

Full papers

- Isolation and Characterization of Cells in Granulomas of Nerves of Leprosy Patients. **Kehrer D.F.S.**, Narayanan R., Malaviya G., Girdhar B. Acta Leprologica 7; 7-13, 1989
- A simple method for the quantification of portosystematic shunting (PSS) in patients with portal hypertension. Bodvarsson A., Verdegaal W.J., Slaats E., Geraedts A.A.M., **Kehrer D.F.S.**, Silberbusch J. Neth J Med 43; 204-209, 1993
- CO-intoxication, treatment with or without hyperbare oxygen-therapy. **Kehrer D.F.S.**, NIGA 96-97
- Fever from the tropics, think of measels. **Kehrer D.F.S.** NIGA 97-98
- Liposomal lurtotecan (NX211); determination of total drug levels in human plasma and urine by reversed-phase high-performance liquid chromotography, Loos W.J., **Kehrer D.F.S.**, Brouwer E., Verweij J., de Bruijn P., Hamilton M., Gil S., Nooter K., Stoter G., Sparreboom A. J. Chromatotgraph. B 738; 155-163, 2000
- Factors involved in prolongation of the terminal disposition phase of SN-38, clinical and experimental studies. **Kehrer D.F.S.**, Yamamoto W., Verweij J., de Jonge M.J.A., de Bruijn P., Sparreboom A. Clin. Cancer Res 6(9); 3451-3458, 2000
- Liquid-chromotographic determination of ketoconazole, a potent inhibitor of CYP3A4-mediated irinotecan (CPT-11) metabolism. de Bruijn P., **Kehrer D.F.S.**, Verweij J., Sparreboom A. J Chromotograph. B 753; 395-400, 2001
- Modulation of camptothecin analogues in the treatment of cancer, a review. **Kehrer D.F.S.**, Soepenberg O., Loos W.J., Verweij J., Sparreboom A. Anti-Cancer Drugs, 12-2; 89-106, 2001
- Modulation of irinotecan-induced diarrhea by co-treatment with neomycin in cancer patients. **Kehrer D.F.S.**, Sparreboom A., Verweij J., de Bruijn P., Nierop C.E., van de Schraaf J., Ruijgrok J., de Jonge M.J.A. Clin Cancer Res, 7(5); 1136-1141, 2001

Abstracts/oral presentations

- Porto-Systemic Shunt estimation in chronic liver desease without clinical signs of cirrhosis. **Kehrer D.F.S.**, Verdegaal W.J., ten Velde J.J.M, Smulders Y.M., Silberbusch J. Neth. J. Med.; 104, 1995.
- Clinical pharmacokinetics of liposomal lurtotecan (NX211) in whole blood, plasma and urine. **Kehrer D.F.S.**, Bos A., Sparreboom A., Loos W.J., Hamilton M., Ptaszynski M., de Vries E., Verweij J. AACR-NCI-EORTC, 337, 1999.
- Pharmacokinetics of irinotecan (CPT-11) in patients treated with neomycin to diminish ß-glucuronidase activity in the intestines. Sparreboom A., **Kehrer D.F.S.**, Verweij J., de Bruijn P., de Jonge M.J.A. AACR-NCI-EORTC; 577, 1999

- Factors involved in prolongation of the terminal disposition phase of SN-38. **Kehrer D.F.S.**, Verweij J., deJonge M.J.A., de Bruijn P., Sparreboom A. Oral presentation AACR; 5171, 2000
- Modulation of irinotecan pharmacokinetics/pharmacodynamics by combined treatment with neomycin. Planting A.J.Th, **Kehrer D.F.S.**, Sparreboom A., de Jonge M.J.A., de Bruijn P., Verweij J. ASCO; 1007, 2000
- Phase 1 study of liposomal lurtotecan (NX 211) administered as a single dose every three weeks. Bos A., **Kehrer D.F.S.**, Sparreboom A., De Vries E., Groen H., Cameron T., Hamilton M., Ptaszynski M.,Verweij J. ASCO; 768, 2000
- Disposition of NX 211 (liposomal Lurtotecan) in cancer patients. Loos, W.J., Verweij, J., **Kehrer, D.F.S.**, de Bruijn, P., de Jonge, M.J.A., Hamiton, M., Sparreboom, A. NCI-EORTC-AACR 239, 2000
- Clinical pharmacodynamics of liposomal lurtotecan (NX 211): urinary excretion predicts hematologic toxicity. Loos, W., **Kehrer, D.F.S.**, Verweij. J., de Vries. E.G.E., Bos, A.M., Hamilton, M., Sparreboom. A., AACR; 551, 2001
- Modulation of CPT-11 metabolism in cancer patients by ketoconazole. **Kehrer D.F.S.**, Verweij J., de Bruijn P., Sparreboom A. AACR; 2897, 2001
- Phase I trial of irinotecan in combination with the farnesyltransferase inhibitor (FTI) R155777. Verweij, J **Kehrer D.F.S.**, Planting A.J.Th., de Jonge M.J.A., Eskens F., Klaren G., de Heus G., Palmer P., Bol C.J., Sparreboom A. ASCO; 319, 2001
- Inhibition of CYP3A4 during irinotecan (CPT-11) chemotherapy can result in potential fatal outcomes. **Kehrer D.F.S.**, Mathijssen R.H.J., Verweij J., de Bruijn P., Sparreboom A. AACR/EORTC/NCI; 404, 2001
- Clinical Pharmacokinetics of irinotecan given in combination with the farnesyltransferase inhibitor ZARNESTRA. **Kehrer D.F.S.**, Sparreboom A., de Jonge M.J.A., de Heus G., Palmer P., Bol C.J., Verweij J. AACR/EORTC/NCI; 449, 2001.

Manuscripts submitted

- Phase I and pharmacologic study of liposomal lurtotecan (NX211): urinary excretion predicts hematologic toxicity. **Kehrer D.F.S.**, Bos A.M.E., Verweij J., Groen H.J., Loos W.J., Sparreboom A., de Jonge M.J.A., Hamilton M.,Cameron T., de Vries E.G.E., JCO. May 2001
- Identification and biological activity of 7-methyl-10,11-ethylenedioxy-20(S)-camptothecin, a photo-degradant of lurtotecan. Loos W.J., Verweij J., **Kehrer D.F.S.**, De Bruijn P., De Groot F.M.H., Hamilton M., Nooter K., Stoter G., and Sparreboom A. Clin. Cancer Res. June 2001
- Irinotecan and CYP3A4 inhibition: dangerous liaisons. **Kehrer D.F.S.**, de Jonge M.J.A., Verweij J., de Bruijn P., Sparreboom A. JCO, July 2001

Manuscripts in preparation

- Phase I and clinical pharmacokinetic trial of irinotecan given in combination with the farnesyl transferase inhibitor R115777. **Kehrer D.F.S.**, Sparreboom, A., de Jonge M.J.A., de Bruijn P., de Heus G., Palmer P., Bol C.J., Verweij J. goal JCO, in preparation, July 2001