Plasma collected from a patient prior to the drug administration did not reveal the presence of endogenous peaks. Concentrations of 9-AC lactone could be readily estimated from protein-free extracts, whereas acidification of plasma samples, re-converting the carboxylate into the corresponding lactone, enabled determination of 9-AC total. The plasma concentration-time curves of 9-AC lactone and 9-AC total forms of a patient treated orally with 2.7 mg of 9-AC, are given in fig. 4. The data indicate that the LLQ's of 50 and 100 pg/ml for 9-AC lactone and 9-AC total, respectively, are sufficient for monitoring drug-plasma levels in samples obtained from patients treated at low oral doses.

In conclusion, two sensitive, selective, accurate and reproducible isocratic reversed-phase HPLC methods have been developed for the analysis of 9-AC lactone and 9-AC total in human plasma. The sample pretreatment procedures are based on single solvent extractions, thereby eliminating the need of laborious solid-phase extraction techniques [12,13]. Compared to previously described assays for 9-AC, our new methods provide equivalent to superior sensitivity with LLQ's of 50 pg/ml for 9-AC lactone and 100 pg/ml for 9-AC total (lactone plus carboxylate). The methodologies described permit the analysis of patient samples, and will be implemented in future investigations on the clinical pharmacology of 9-AC administered at low (oral) doses.

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Chapter 3b

Role of erythrocytes and serum proteins in the kinetic profile of total 9-amino-20(S)-camptothecin in humans

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ABSTRACT

9-Amino-20(*S*)-camptothecin (9-AC) is a water-insoluble topoisomerase-I inhibitor with evident schedule-dependent antitumor activity in preclinical studies. The pharmacokinetic behavior of 9-AC given as a bolus i.v. infusion (1.0 mg/m^2 over 5 min) was recently characterized in 12 patients in a bioavalability study. Remarkable rebound concentrations of 9-AC total drug (i.e. lactone *plus* carboxylate forms) were observed at about 2-3 hours after dosing. *In vitro* experiments indicated that this phenomenon was associated with a substantial uptake of 9-AC lactone by erythrocytes immediately after dosing, and its subsequent release followed by accumulation of 9-AC carboxylate in the plasma compartment mediated by a pH-dependent hydrolysis of the lactone form, which is unable to diffuse across cell membranes. The preferential binding of 9-AC carboxylate to human serum albumin shifts the equilibrium between the lactone and carboxylate forms of 9-AC to the pharmacological inactive carboxylate form.

INTRODUCTION

9-Amino-20(*S*)-camptothecin (9-AC, NSC 603071) is a semisynthetic derivative of the naturally occurring plant alkaloid camptothecin that does not produce hemorrhagic cystitis associated with the parent compound [1]. The mechanism of action of 9-AC is based on inhibition of topoisomerase-I, an intranuclear enzyme which relaxes supercoiled DNA by creating single strand DNA breaks which are subsequently religated [2]. In preclinical studies, 9-AC demonstrated significant activity, (including cures) in mice xenografted with human carcinomas resistant to common antineoplastic agents [3]. These animal studies further demonstrated that drug efficacy is critically dependent on the duration of exposure, frequency of administration and plasma levels of the drug. On the basis of these data, several clinical trials have been performed with the drug administered using various continuous i.v. infusion schedules [4-9]. Currently, there is considerable interest to explore alternative routes and schedules of 9-AC administration, e.g. oral [10-12] or daily i.v. bolus administration [13], to facilitate the development of more prolonged dosing that may be required to optimize antitumor activity.

Pharmacokinetic studies with camptothecin analogues, including 9-AC, were previously shown to be complicated by a chemical, pH-dependent instability of the terminal α-hydroxy-δ-lactone ring (Fig. 1), generating a ring-opened carboxylate, which is over 1000-fold less active as an inhibitor of topoisomerase-I [14]. The clinical pharmacokinetics of 9-AC has been studied extensively in patients receiving the drug by i.v. infusion over 24 or 72 hours [4,5,7,9,15]. These studies showed that only about 10% of the total plasma 9-AC circulated in the active lactone form, which is considerably lower than that reported for topotecan [16] and the irinotecan metabolite SN-38 [17]. It has been

suggested, that this is related to differential affinity of the carboxylate forms of camptothecins for human serum albumin, causing a shift in the equilibrium hydrolysis within the systemic circulation [18].

Recently, we reported the pharmacokinetics of 9-AC lactone and carboxylate after bolus i.v. administration in 12 cancer patients participating in a bioavailability study [19]. In the present work, we evaluate the kinetic profile of the total 9-AC concentration, revealing a new feature of 9-AC disposition in humans, which is characterized by a significant rebound peak in the plasma profile. The role of erythrocytes and serum proteins in the kinetic profile of total 9-AC is described in this report.

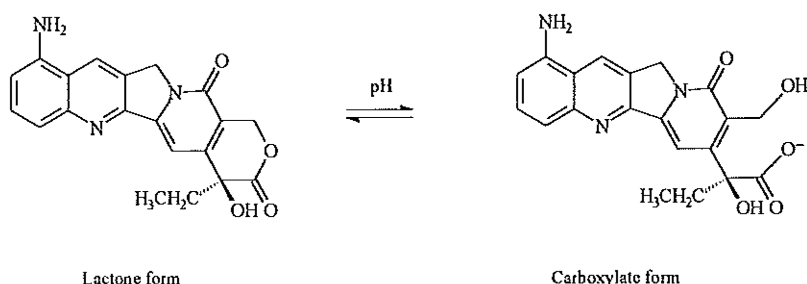

Fig. 1: Chemical structures and pH-dependent interconversion of the lactone and carboxylate forms of 9-aminocamptothecin.

MATERIALS AND METHODS

Chemicals and reagents

Pure reference standards of 9-AC (batch: 93L07A) and camptothecin (batch: 93K05A were provided by Pharmacia & Upjohn (Milan, Italy). Perchloric acid was obtained from Baker (Deventer, The Netherlands) as a 70% (v/v) aqueous solution. Human and murine serum albumin were purchased as essentially fatty acid free lyophilized powders from Sigma Chemicals Co. (St. Louis, MO, USA). All other reagents were of analytical grade or higher, and originated from Rathburn (Walkerburn, UK). Samples of human plasma and whole blood were obtained from healthy volunteers. HPLC grade water was prepared in-house using the Milli-Q UF Plus system from Millipore (Bedford, MA, USA).

Clinical pharmacokinetics

The pharmacokinetic profiles of the lactone and carboxylate forms of 9-AC after bolus i.v. administration were previously described by Sparreboom et al [19]. Briefly, 12 patients enrolled in a bioavalability study in which they received the lactone form as a single i.v. bolus of 1.0 mg/m^2. Quantitative determination of the lactone and total drug concentrations were performed in serial plasma samples obtained upto 55 h after dosing using a reversed-phase HPLC method as described earlier by Loos et al [20].

***In vitro* stability of 9-AC**

The rate of hydrolysis of 9-AC lactone was monitored in PBS, 4% (w/v) solutions of human and murine serum albumin in saline, and in heparinized human whole blood. All matrices were incubated in triplicate with 1.0 μg/ml of 9-AC lactone at 37°C, and 250-μl samples were taken at 0, 0.5, 1, 2, 4, 7.5, and 24 h after start of the incubation. The samples were diluted 2.5 to 50-fold in a pool of drug-free human plasma and analyzed for the intact 9-AC lactone and 9-AC total forms by HPLC as described by Loos et al. [20].

In a separate series of experiments, extracellular 9-AC total concentration-time profiles were monitored in samples of human whole blood, erythrocyte-rich PBS, a leukocyte buffy coat suspension, and platelet-rich plasma. Platelet-rich plasma with very little contamination from erythrocytes and leukocytes was prepared by centrifugation at 200×*g* for 20 min at room temperature [21]. Kinetic runs were initiated by addition of 9-AC lactone at 37°C to provide an initial concentration of 100 ng/ml. Aliquots were withdrawn periodically, processed to cell free samples by centrifugation at 15,000×*g* for 2 min (4°C), and analyzed by HPLC as described above.

Accumulation of 9-AC in cell cultures.

The human ovarian and colon carcinoma cell lines IGROV-1 and WIDR, and the non-malignant African green monkey kidney cell line VERO were cultured in Dulbecco's modified Eagle's Medium (DMEM; GibcoBRL, Life Technologies B.V., Breda, The Netherlands), supplemented with 10% of heat-inactivated fetal calf serum (Hyclone, Logan, UT), 10 m*M* of sodium carbonate, 2 m*M* of L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Life Technologies) in a humidified atmosphere of 5% CO_2 at 37°C. All cell lines were grown to 80 to 90% confluence in 6-well culture plates (Greiner, Alphen a/d Rijn, The Netherlands), and incubated in triplicate with 1.0 μg/ml of 9-AC lactone or 9-AC carboxylate for 10 min. The cells were washed rapidly three times with ice-cold saline, and harvested by scraping in 500 μL of water. An aliquot of 250 μl was used for determination of 9-AC total drug concentrations by HPLC, and 10 μl were used for the determination of the total protein content by a modification of the Bradford dye-binding method [22].

RESULTS

Clinical pharmacokinetics

As described earlier [19], the plasma concentration-time profiles of the lactone and carboxylate forms of 9-AC were remarkably similar for the 12 patients studied, with a very short initial half-life time of the lactone form of approximately 6.5 min. The overall estimated lactone to total drug ratio in plasma was 9.1±3.4%, indicating a rapid and substantial conversion to the carboxylate species.

Evaluation of the plasma profile of total 9-AC (i.e. lactone plus carboxylate) revealed a very pronounced secondary peak in all 12 patients, with a maximum of the total occurring at 2-3 hours after drug administration (Fig. 2).

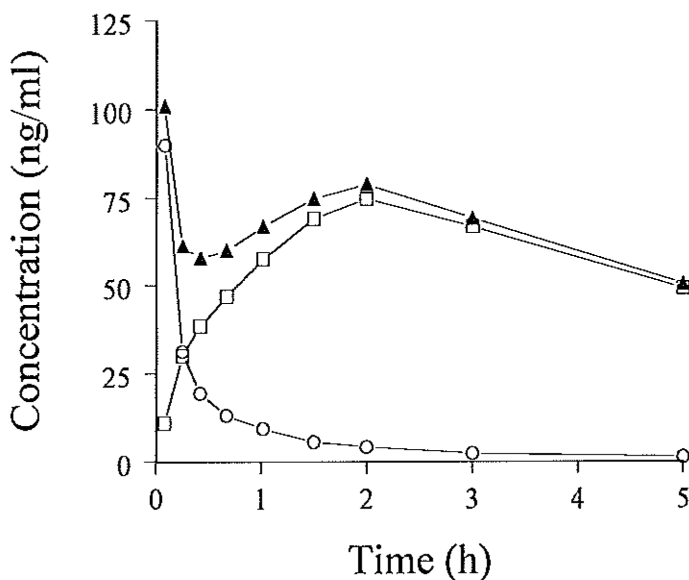

Fig. 2: Representative plasma concentration-time profile of 9-AC lactone (O), carboxylate (□) and total drug (i.e. lactone plus carboxylate forms;▲) in a single patient after i.v. drug administration of 1.0 mg/m^2

***In vitro* studies**

In order to gain insight into the pharmacologic mechanisms involved in this rebound peak phenomenon, various *in vitro* stability studies of 9-AC were performed. Equilibrium distribution ratios for accumulation of 9-AC into erythrocytes were found to change dramatically with time for 9-AC lactone concentrations in the therapeutically relevant range of 0.1 to 1.0 μg/ml (Fig. 3).

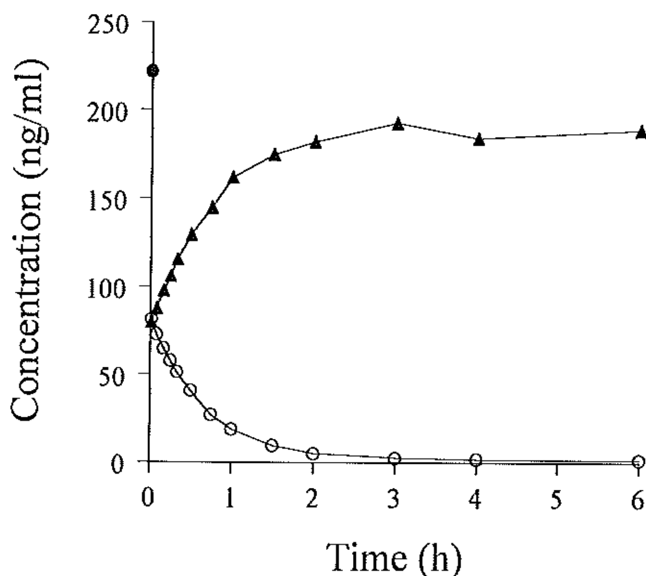

Fig. 3: Extracellular concentration-time profiles of 9-AC lactone lactone (O) and 9-AC total drug (▲) following incubation of 9-AC lactone at 100 ng/ml in human whole blood. The closed circle on the ordinate indicates the initial 9-AC total extracellular concentration estimate, based on a hematocrit of 0.45.

Under the same experimental conditions, there was no substantial drug accumulation in human platelets or peripheral leukocytes that may have affected the kinetic behavior of 9-AC (total drug) in human plasma (data not shown). Measurement of extracellular 9-AC total drug following incubation of 9-AC lactone in human whole blood demonstrated a rapid fall in concentration, caused by drug

accumulation in erythrocytes, followed by a continuous rise until an apparent steady state was established at approximately 2-3 hours (Fig. 3), similar to that observed in our patients. At steady-state, the 9-AC (total drug) plasma to erythrocyte concentration ratio was estimated to be about 100:1. This result can be understood by considering that the large fraction of 9-AC lactone taken up initially into erythrocytes (~25-30%) will redistribute to the plasma water, followed by dissociation of the lactone moiety due to the physiologic pH and the presence of serum proteins (Table 1). Under *in vitro* conditions in PBS, 10.7±0.4% of 9-AC was in the lactone form at equilibrium. The addition of human serum albumin, however, was found to further shift the lactone to carboxylate equilibrium dramatically in favor of the latter due to a higher affinity of the carboxylate form, with less than 1% of total drug remaining as lactone. In whole blood, 0.8±0.2% of 9-AC was in the lactone form at equilibrium, which is not significantly different from human serum albumin solution and human plasma. The fact that only the intact lactone form of 9-AC can diffuse across cell membranes further contributed to the subsequent accumulation of the carboxylate species (Table 2). Drug accumulation in the various cell lines tested after incubation with the carboxylate form was only approximately 5% in comparison with the accumulation during exposure to the lactone form. However, the cellular accumulation is seriously influenced by the lactone-carboxylate interconversion during incubation, as only 3.5±0.15% (mean±SD) of extracellular carboxylate is converted into lactone after 10 min at 37°C, compared to 38.2±2.07% of lactone into carboxylate.

Table 1: Stability of 9-AC lactone at equilibrium in various media[a]

Matrix	$t_{1/2}$[b] (min)	% as lactone at equilibrium	pH
Phosphate buffered saline	25.8±0.31	10.7±0.42	7.4
Human serum albumin	37.9±1.9	0.63±0.10	7.0
Murine serum albumin	244±17.5	35.0±6.2	7.0
Human whole blood	23.6±0.48	0.81±0.21	7.4
Human plasma	12.2±0.72	1.3±0.50	7.4

a: All matrices were incubated in triplicate with 1.0 µg/ml of 9-AC lactone at 37°C, with serial samples taken upto 24 h.
b: Abbreviation: $t_{1/2}$, half-life.

Table 2: Cellulair accumulation of 9-AC lactone and 9-AC carboxylate in various cell lines[a]

Cell line	9-AC lactone (ng/ml protein)	9-AC carboxylate (ng/ml protein)	C/L[b] (%)
IGROV-1	29.9 ± 1.97	1.5 ± 0.24	5.1
VERO	22.3 ± 1.65	1.2 ± 0.30	5.4
WIDR	32.5 ± 1.08	1.8 ± 0.10	5.5

a: All cell lines were incubated in triplicate with 1.0 μg/ml of 9-AC lactone or 9-AC carboxylate for 10 min at 37°C.
b: Carboxylate to lactone concentration ratio

DISCUSSION

Recently we have described the pharmacokinetics of 9-AC in a cohort of patients that received the drug by bolus i.v. administration [19]. The lactone hydrolysis was rapid with greater than 90% conversion to the pharmacologically inactive, ring-opened carboxylate form within 3 hours following a 5-min infusion, which is similar to earlier findings [5,15]. The pharmacokinetic profile of 9-AC total drug (this report) was characterized by the presence of a major secondary peak, and was shown to be caused by an unusual mechanism involving initial uptake of 9-AC lactone in erythrocytes, followed by the progressive accumulation of the carboxylate form of the drug in plasma. Pharmacokinetic studies performed during clinical trials of 9-AC administered as a continuous i.v. infusion have consistently failed to recognize this behavior [4,5,7,9,15]. This can be explained by the fact that the frequency of sampling during the first several hours after infusion was not only less intensive, but the steady state levels of total drug at the maximum tolerated dose were more than 10-fold lower than the peak levels in the present report.

The rate of uptake of 9-AC lactone by erythrocytes *in vitro* was too rapid to be reliably estimated, with an initial uptake half-life in the order of 30 s or less from phosphate buffered saline (to a hematocrit of ~0.45) or human whole blood. This indicates that the rate of exchange between red cells and plasma water is sufficiently rapid to be effectively instantaneous on the time scale of disposition of 9-AC. However, the rate of 9-AC lactone uptake *in vivo* is clearly influenced by the extensive binding of 9-AC carboxylate to proteins, principally serum albumin, causing a gradual depletion of drug available for cellular distribution. Thus, the apparent contradiction between the

high plasma to blood cell concentration ratio of 9-AC total drug and the high initial accumulation of the lactone form into erythrocytes is due to a compensating effect of protein binding of the carboxylate form following hydrolysis of the α-hydroxy-δ-lactone function, thereby trapping the drug in the plasma compartment. The net effect is presumably that early after drug dosing, erythrocytes act as a depot from which the plasma 9-AC lactone is supplied, followed by accumulation of the 9-AC carboxylate in plasma. While we did not test this hypothesis directly, our *in vivo* data would appear to support this notion. In particular, our finding that 9-AC carboxylate did not diffuse across cell membranes is consistent with the significantly reduced volume of distribution for this species in our patients [19], and suggests a preferential cellular uptake of the lactone accompanied by accelerating predominance of the carboxylate in plasma.

Previous studies indicated that this unusual disposition feature may not be unique for 9-AC, as this rebound phenomenon has also been described for the related compound, irinotecan. The initial observation of this behavior was described by Rivory et al. [17], and similar data have been generated from numerous patients treated with irinotecan, although the authors surmized that it might be related to enterohepatic recirculation [23-25]. However, the observation of rebound concentrations of irinotecan and its metabolite SN-38 are not as distinct as those seen with 9-AC in the present study, and are apparently only observed with the drug administered to patients using short i.v. infusion schedules (i.e. $\leq$30 min). This is probably because drug concentrations will often not be significant at low plasma concentrations to ascertain the secondary peak, and it is only as the plasma concentration increases locally, e.g. following an i.v. bolus injection, that erythrocytes carry a physiologically relevant load [26]. In case of SN-38, the situation is also complicated by the occurrence of enterohepatic cycling following biliary secretion of the hydrophilic C10-glucuronic acid conjugate, which is hydrolyzed by bacterial β-glucuronidase in the intestines [27]. Another possible explanation for the discrepancy between camptothecins in the rebound phenomenon may come from the differences in protein binding of the lactone and carboxylate species for the different camptothecins. It has been described previously that the binding affinity of both drug forms for human serum albumin is an important determinant in the marked differences in the lactone to carboxylate ratios between drugs [18]. For 9-AC and the parent drug camptothecin, serum protein binding of the carboxylate form is highly favored over the closed ring form, and an equilibrium favoring the carboxylate form is rapidly established. In case of 9-AC, this is probably the main reason for the extremely low lactone to total drug AUC ratio (i.e. <10%). For topotecan, irinotecan and SN-38 these ratios are 36%, 38% and 53%, respectively [16,17]. Therefore, it is reasonable to assume that similar effects will be less pronounced in case of topotecan and irinotecan. The absence of the secondary peak in the 9-AC total plasma profile in rodents [28] most likely also relates to differences in lactone to carboxylate ratios compared to humans; at equilibrium, the amount of 9-AC remaining in the lactone form in human plasma is 1.30±0.50%, whereas in mouse plasma or murine serum albumin solution, approximately 35% is present as the lactone form (Table 1).

In conclusion, we have shown that a major rebound peak in the plasma profile of 9-AC arises as the result of a balance between extensive erythrocyte uptake of the lactone form and extensive serum protein binding of the carboxylate form within the systemic circulation, resulting in a shift in equilibrium over time. These data support the idea that 9-AC is an exceptionally dynamic drug in biological systems, with its pharmacokinetic profile influenced strongly by hydrolytic processes as well as by differential cellular distribution and protein binding of the lactone and carboxylate forms.

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Chapter 3c

Clinical pharmacokinetics of encapsulated oral 9-aminocamptothecin in plasma and saliva

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ABSTRACT

Objective To study the pharmacokinetics and pharmacodynamics of the novel topoisomerase I inhibitor and antitumor agent, 9-amino-20(*S*)-camptothecin (9-AC), in patients with solid tumors after repeated oral dosing.

Methods Thirty-two cancer patients received oral 9-AC formulated in capsules with polyethylene glycol-1000 as excipient at doses that ranged from 0.25 to 1.5 mg/m^2/day. Serial plasma and saliva samples were obtained on days 1 and 6 or 8 of the first cycle and analyzed for the lactone and carboxylate forms of 9-AC by HPLC.

Results 9-AC demonstrated linear and dose-independent pharmacokinetics, with extremely small intrapatient kinetic variability (coefficient of variation: <10%). However, interpatient variability in plasma pharmacokinetics was large (coefficient of variation: 99%). The relative extent of lactone to carboxylate interconversion was large (>90%), and predictable from individual pretreatment serum albumin values (p=0.0099). The 9-AC concentration ratio in plasma and saliva was strongly patient dependent, and highly variable around a mean value of 1.4 , suggesting that saliva is an unreliable matrix for kinetic monitoring. The area under the curve of the lactone form of 9-AC was significantly correlated with the dose-limiting hematological toxicity (p<0.001).

Conclusion Our data indicate that the large interindividual pharmacodynamic variability in response to 9-AC is mainly caused by a variability in kinetic characteristics, suggesting that a kinetic-dynamic guided study design is warranted in future clinical investigations.

INTRODUCTION

In the early 1970s, camptothecin, a plant alkaloid extract from the bark and wood of the Chinese tree *Camptotheca acuminata*, was demonstrated to possess antineoplastic activity [1]. Analogs of camptothecin belong to a family of anticancer agents with a unique mechanism of action, which is based on reversible inhibition of DNA topoisomerase I [2,3].

Despite the cytotoxicity of the compound further development was halted because of a number of severe and unpredictable side-effects observed in early clinical trials [4-6]. The subsequent search for less toxic analogs of camptothecin resulted in the discovery of irinotecan, topotecan, 9-amino-20(S)-camptothecin (9-AC), 9-nitrocamptothecin, DX-8951f and GI147211. The first two of these, irinotecan and topotecan, were recently registered for the treatment of colorectal and ovarian cancer, respectively.

In aqueous solutions, camptothecins are unstable and undergo a rapid, pH-dependent, non-

enzymatic hydrolysis of the terminal lactone ring to form the more water-soluble, ring-opened carboxylate form (Fig. 1) [7]. The presence of the intact terminal lactone ring is thought to be essential for the topoisomerase I inhibition [8]. The closed lactone ring predominates at acidic pH, whereas in human plasma, the equilibrium between these two species greatly favors formation of the carboxylate form, partly because of the physiologic pH and the preferentially binding of this form to albumin [9,10]. The ratio of the lactone form to the total drug concentration at steady state in plasma is different for each camptothecin analog, which might have important pharmacokinetic and pharmacodynamic implications.

In preclinical studies 9-AC demonstrated activity against human colon, breast, prostate, non-small cell lung cancer and melanoma xenografts 11-14]. Preclinical *in vivo* data suggested that duration of exposure to 9-AC lactone above a certain threshold concentration (10 n*M*) and frequency of administration were essential for antitumor activity [15,16].

Based on its preclinical activity, 9-AC appeared to merit evaluation as an antineoplastic agent. To mimic the preclinical studies, initial Phase I studies using the intravenous formulation of 9-AC focused on schedules with prolonged infusion duration of 24-120 hr [17-21], or a continuous infusion for 21 days every 4 weeks [22]. Pharmacokinetic data obtained during these studies showed marked interpatient variability. Steady state plasma concentrations of 9-AC lactone greater than 10 n*M* were achieved only in the Phase I study of the 24-hour infusion of 9-AC at the dose recommended for further Phase II studies (i.e. 1.65 mg/m^2). When 9-AC was administered as a 72-hr infusion once every 2 or 3 weeks, the maximal tolerated dose was 35-54.2 μg/m^2/hr. The dose limiting toxicity consisted of neutropenia in combination with thrombocytopenia and correlated to the steady state 9-AC lactone concentration.

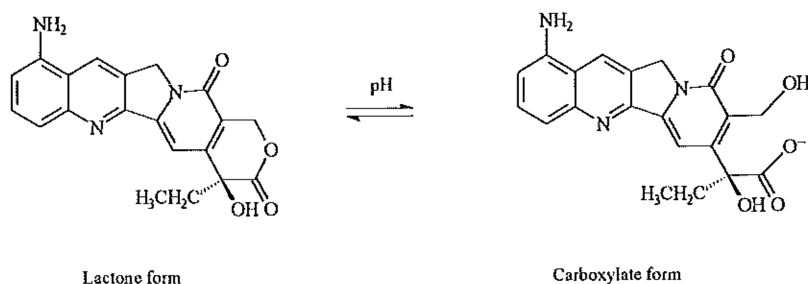

Fig. 1: Chemical structures and pH-dependent interconversion of the lactone and carboxylate forms of 9-aminocamptothecin.

Recently, we reported that 9-AC demonstrated rapid absorption in humans after oral delivery with an overall bioavailability of approximately 50% [23]. In the present report, we present a comprehensive analysis of the plasma pharmacokinetics of the lactone and carboxylate forms of 9-AC in cancer patients receiving the drug orally over a wide range of dose levels, with special focus on pharmacokinetic-pharmacodynamic characteristics. In order to assess the clinical usefulness of salivary monitoring of 9-AC for kinetic modeling, paired plasma and coinciding unstimulated saliva samples were collected in a limited number of patients.

MATERIALS AND METHODS

Patient population

The patients, from whom pharmacokinetic curves were obtained, participated in an oral bioavailability study of 9-AC and/or in a Phase I trial of oral 9-AC administered daily for 7-14 consecutive days every 3 weeks. Treatment plans and detailed clinical profiles have been documented elsewhere [23]. Eligibility criteria included a histologically or cytologically confirmed diagnosis of a solid malignant tumor not amenable to established forms of treatment. All patients had an adequate hematopoietic (absolute peripheral granocyte count ≥2.0 x 10^9/L and platelet count ≥100 x 10^9/L), hepatic (bilirubin within normal limits, and serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase ≤2.5 times normal limit) and renal (serum creatinine <133 *μM*) function. Other eligibility criteria included the following: age between 18 and 75 years; Eastern Cooperative Oncology Group performance status ≤2; estimated life expectancy ≥12 weeks; no previous anticancer therapy for at least 4 weeks (6 weeks for nitrosoureas or mitomycins); no previous therapy with other camptothecins and/or intensive ablative regimens. Specific exclusion criteria included significant gastrointestinal dysfunction that could alter absorption or motility, and chronic treatment with corticosteroids. Concomitant administration of H_2-antagonists, antacids, proton-pump inhibitors and non-steroidal anti-inflammatory drugs were avoided. All patients gave written informed consent before study entry.

Treatment plan and dose escalation

The oral formulation of 9-AC was supplied by Pharmacia & Upjohn (Nerviano, Italy) as hard gelatin capsules that contained 0.10, 0.25 or 1 mg of the active drug and polyethylene glycol-1000 (PEG1000) as excipient [23], and were stored at room temperature.

A detailed description of the preparation and rationale for composition of the formulation has been described earlier [23]. Patients received 9-AC orally with 150-200 mL of water at dose levels of 0.25, 0.40, 0.60, 0.84, 1.0 or 1.1 mg/m^2/day for 7 or 14 consecutive days, or as a single dose of 1.5 mg/m^2.

Intrapatient dose escalation in the phase I trial was not permitted. Weekly evaluation of the patients included a clinical history, physical examination, toxicity assessment according to common toxicity criteria (CTC), and serum chemistry. A complete blood cell count with differentiation was determined twice weekly.

Sample collection and drug analysis

Serial blood samples were collected in heparinized tubes from an indwelling venous catheter at 0, 0.33, 0.67, 1, 1.5, 2, 3, 5, 7.5, 11 and 24 hours after administration on days 1 and 6 or 8 of the first treatment course. In a limited number of patients, unstimulated saliva samples were obtained at coinciding time points. In one patient with a malignant pleural effusion additional pleural fluid sampling was performed to assess the influence of the pleural effusion on the plasma pharmacokinetics. Immediately after sampling, aliquots of plasma (separated at 4°C), pleural fluid and saliva were frozen at -80°C, and analyzed later for 9-AC lactone and 9-AC total drug (i.e. lactone *plus* carboxylate) with use of a validated reversed-phase high-performance liquid chromatographic method as described previously [24]. Drug concentrations in patient plasma samples were calculated using interpolation of the corresponding regression analysis. Specimens with drug levels exceeding the upper range of the calibration curve were reanalyzed upon appropriate dilution with drug-free plasma. Saliva and pleural fluid samples were diluted 4-fold in drug-free plasma and analyzed with use of the same analytical assay, with minor modifications. The lower limits of detection of the assays were 0.05 ng/mL in plasma and 0.4 ng/mL in pleural fluid and saliva.

Data analysis

Plasma-concentration data were analyzed by both non-compartmental and compartmental analysis using the Siphar version 4.0 software package (SIMED, Créteil, France). The model-independent pharmacokinetic parameters included the maximum plasma concentration (C_{max}) and the time to reach the peak concentration (t_{max}). Initial parameter estimates were obtained by an automated peeling algorithm based on the Powell method to three compartments, which yielded the best statistical fit as determined by Akaike's information criterion and the *F*-test. The AUC values were calculated based on the best fitted curve, as were the disposition half-lives ($t_{1/2}$). The apparent absorption rate constant (k_a) was obtained through numerical point-area deconvolution. Relationships between the AUC and pharmacodynamic outcome were evaluated with (log-)linear and (non-)sigmoidal-maximum effect modeling using Siphar and NCSS version 5.0 (Dr.Jerry Hintze, East Kayesville, UT).Within individual patients, myelosuppression was described as the continuous variable, consisting of percentage decrease in white blood cell count (WBC), absolute neutrophil count (ANC) and platelet count (PLT). The relative hematological toxicity was defined as : % decrease = (pretherapy value-nadir value)/(pretherapy value)*100. Only the first course of each patient was taken into consideration

to avoid potentially confounding bias due to cumulative toxicity. All data were fitted to a sigmoidal maximum effect (E_{max}) model based on the modified Hill equation, as follows: $E=E_0+E_{max}*[(KP^{\gamma})/(KP^{\gamma}+KP_{50}^{\gamma})]$. In this equation, E_0 is the minimum reduction possible, fixed at a value of 0, E_{max} is the maximum response, fixed at 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 describing the sigmoidicity of the curve. Models were evaluated for goodness of fit by minimisation of sums of the squared residuals and by reduction of the estimated coefficient of variation for fitted parameters. Significance of the relationships were assessed by construction of contingency tables with subsequent χ^2 analysis.

Table 1: Patient characteristics

Characteristic		No. of patients
No. entered		32
No. assessable for toxicity		30
Age, years		
Median	59	
Range	29-74	
Sex		
Female		14
Male		16
Performance status		
Median	1	
Range	0-2	
Tumor type		
Colorectal		14
Ovarian		5
Sarcoma		2
Mesothelioma		2
Lung (non-small cell)		2
Miscellaneous		5
Previous treatment		
Chemotherapy		16
Radiation		2
Chemotherapy and radiation		10
None		2

RESULTS

Demographic characteristics of all 32 patients who had blood sampling for pharmacokinetic analysis are shown in Table I. Ten patients completed the oral bioavailability study and subsequently participated in the phase I and pharmacokinetic study of oral 9-AC. Hence, pharmacokinetic data were obtained in 42 courses. Plasma pharmacokinetics could not be determined in 1 course on day 1 and in 2 courses on day 8 as a result of limited sample availability or significant chromatographic interference in the drug assay by an unknown compound. Twenty-seven patients were assessable for pharmacodynamic analysis and 30 patients were assessable for toxicity.

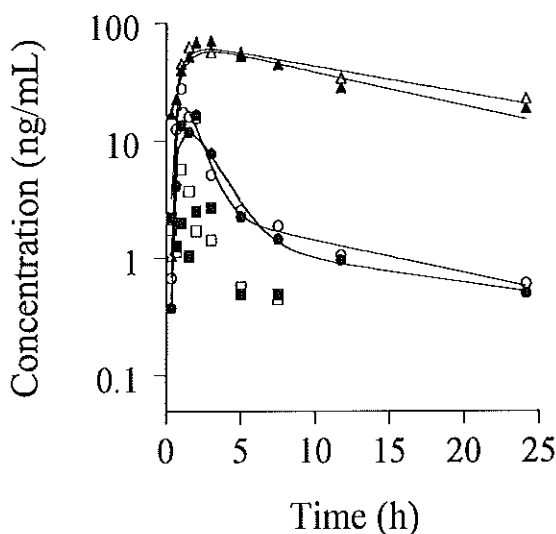

Fig. 2: Representative concentration-time profiles of 9-AC lactone (circles) and 9-AC total (triangles) in plasma and in saliva (rectangle) measured on day 1 (open symbols) and day 8 (closed symbols) of the first treatment course in a single patient following oral administration of 9-AC at a dose level of 0.84 mg/m^2/day in a daily-times fourteen schedule. All pharmacokinetic curves were fitted to a tri-exponential equation assuming a three-compartment modal for the distribution and elimination of the drug.

The plasma concentration-time profiles of 9-AC lactone and 9-AC carboxylate were similar for all patients studied, with representative examples shown in Fig. 2. The pharmacokinetics of both species of 9-AC could be best described with a three-compartment model. The kinetic parameters obtained by means of this model are presented in Tables II and III. The absorption of 9-AC lactone after oral administration was rapid with a lag time of 0.29±0.07 hr (mean ± SD; n=41), maximum peak drug levels at 0.99±0.12 hr, and a mean absorption rate constant of 3.03±0.90 h^{-1}. In the first plasma samples the ring-opened carboxylate form of 9-AC was already detectable. Maximal plasma concentrations of 9-AC carboxylate were reached at 2.41±0.64 hr after dosing. Eventually, the 9-AC carboxylate accounted for 91.1±2.11% of 9-AC total drug concentrations, indicating a clear predominant conversion of lactone to carboxylate. Elimination of 9-AC from the central plasma compartment was characterized by a decay in an apparent tri-exponential manner based on conventional compartment modeling using weighed least-squares analysis with a weighting factor of $1/Y$. The mean values for the linear segments of 9-AC lactone were $t_{1/2}(\alpha)$: 0.26 hr (range, 0.13-0.38 hr), $t_{1/2}(\beta)$: 0.78 hr (range, 0.54-1.13 hr) and $t_{1/2}(\gamma)$: 7.47 hr (range, 3.66-12.6 hr). No significant quantitative differences were observed between the decay kinetics of 9-AC lactone and 9-AC carboxylate.

Table 2: Model-dependent pharmacokinetics of 9-AC_{lac} after oral drug administration of 9-AC.

Dose (mg/m^2/day)	0.25	0.40	0.60	0.84
n	6	3	4	6
k_a (1/h)	ND	ND	ND	ND
t_{lag} (h)	0.33±0.13	0.32±0.05	0.38±0.14	0.28±0.05
t_{max} (h)	1.06±0.35	0.79±0.15	0.94±0.11	0.98±0.27
C_{max} (ng/mL)	2.09±0.82	7.94±7.39	3.43±0.60	4.61±1.51
$t_{1/2}(\alpha)$ (h)	0.25±0.20	0.13±0.11	0.23±0.07	0.24±0.11
$t_{1/2}(\beta)$ (h)	0.68±0.40	0.82±0.33	0.54±0.20	0.73±0.33
$t_{1/2}(\gamma)$ (h)	5.22±4.22	8.65±1.25	3.66±0.55	10.3±6.77
$AUC_{0-\infty}$ day 1 (ng.h/mL)	6.88±4.76	16.2±14.5	8.15±0.76	12.9±3.97
$AUC_{0-\infty}$ day 8 (ng.h/mL)	9.48±3.85	17.4±15.5	8.69±0.70	11.7±2.98
intra %CV of AUC	7.31	7.31	4.44	9.74
inter %CV of AUC	69.2	89.5	89.2	30.7
% of 9-AC_{total} AUC	7.18±2.33	12.9±4.20	8.76±3.49	6.98±1.72

The kinetic data and recorded AUC values for the following days of administration were similar to those achieved the first day in the same patient (Tables 2 and 3). Hence, the resulting intrapatient variability in AUC and peak drug levels, expressed as the coefficient of variation, was extremely small and averaged 8.67% for 9-AC lactone and 10.9% for 9-AC carboxylate. The interpatient variability in the observed pharmacokinetics, however, was large, with coefficients of variation in AUC values as high as 89.5% for 9-AC lactone and 99.0% for 9-AC carboxylate.

Over the total dose range studied, 9-AC lactone and 9-AC total demonstrated linear and dose-independent pharmacokinetics (Figs. 3*A* and *B*, respectively). No significant relationship was observed between the AUCs of 9-AC total and that of the pharmacologically active species, 9-AC lactone (not shown). The interpatient variation in the equilibrium ratio of 9-AC lactone and 9-AC carboxylate could be explained in part by a individual differences in pretreatment serum albumin levels, for which a significant correlation with the AUC ratio of 9-AC lactone and 9-AC carboxylate could be demonstrated (r=0.471, p=0.0099). This finding clearly indicates that separate monitoring of 9-AC lactone and 9-AC carboxylate concentrations is mandatory to relate drug levels to pharmacodynamic outcome in patients treated with oral 9-AC.

1.0	1.1	1.5
7	3	12
ND	ND	3.03±0.90
0.29±0.05	0.17±0.13	0.24±0.13
0.99±0.29	1.00±0.01	1.17±0.33
11.0±7.46	12.2±6.95	6.82±2.98
0.24±0.09	0.36±0.07	0.38±0.21
0.76±0.51	0.82±0.16	1.13±0.59
5.10±2.68	6.76±2.26	12.6±4.20
31.2±19.1	49.3±29.7	31.9±14.3
31.2±27.0	48.5±23.8	ND
4.96	6.48	ND
61.1	60.3	44.8
10.2±4.94	8.67±3.22	7.30±3.22

Abbreviations: n, number of patients; k_a, absorption rate constant; t_{lag}, lag time; t_{max}, time to peak plasma levels; C_{max}, maximum plasma concentrations; $t_{1/2}(i)$, half-life of the i-th disposition phase; AUC, area under the plasma concentration *versus* time curve; CV, coefficient of variation.

Table 3: Model-dependent pharmacokinetics of 9-AC$_{car}$ after oral drug administration of 9-AC.

Dose (mg/m^2/day)	0.25	0.40	0.60	0.84
n	6	3	4	6
t_{lag} (h)	0.41±0.20	0.34±0.04	0.39±0.13	0.31±0.02
t_{max} (h)	2.60±1.19	2.20±0.601.6	3±0.23	2.01±0.49
C_{max} (ng/mL)	9.43±3.50	11.8±5.76	14.5±6.93	15.2±4.88
$t_{1/2}(\alpha)$ (h)	0.55±0.50	0.40±0.30	0.20±0.20	0.18±0.26
$t_{1/2}(\beta)$ (h)	1.78±0.67	1.12±0.44	1.00±0.69	1.07±0.55
$t_{1/2}(\gamma)$ (h)	7.50±3.96	6.59±2.82	4.98±1.74	9.61±3.73
$AUC_{0-\infty}$ day 1 (ng.h/mL)	90.8±48.2	89.0±49.4	102±45.1	174±39.4
$AUC_{0-\infty}$ day 8 (ng.h/mL)	177±127	137±105	83.9±34.1	162±36.3
intra %CV of AUC	13.9	27.7	1.63	8.52
inter %CV of AUC	53.1	55.5	44.3	22.6
% of 9-AC$_{total}$ AUC	92.8±2.33	87.1±4.20	91.2±3.49	93.0±1.72

Salivary drug monitoring was evaluated as an option for determining the AUC of 9-AC lactone. The 9-AC lactone concentration ratio in plasma and unstimulated saliva was strongly patient-dependent and highly variable around a mean value of ~1.4, suggesting that saliva is an unreliable matrix for pharmacokinetic analysis of this drug (Fig. 2).

To determine the impact of a pleural effusion on the pharmacokinetics of 9-AC, plasma and pleural effusion samples were obtained for drug analysis in a single patient with a malignant pleural effusion. The mean pleura versus plasma concentration ratio of 9-AC lactone was 4.95%±2.32 (mean ± SD; range, 0.7-6.8%).These data indicate that pleural effusion does not constitute a major pharmacokinetic compartment for this drug.

The pharmacokinetic data obtained from 27 patients were plotted against the percentage decrease in white blood cell count (WBC), platelet count (PLT) and absolute neutrophil count (ANC), at nadir relative to the pretreatment value. Four different models, based on linear, log-linear, maximum effect (E_{max}), and sigmoidal E_{max} fitting, were compared for their ability to describe the data. Using sigmoidal E_{max} modeling of the pharmacokinetic and hematological toxicity data significant correlations between the AUC of 9-AC lactone and the percentage decrease in WBC (r=0.86; p<0.001; Fig. 4*A*), percentage decrease in PLT (r=0.83; p<0.001; Fig. 4*B*) and percentage decrease in ANC (r=0.66; p<0.001; Fig. 4*C*) could be demonstrated. In addition, the worst observed

1.0	1.1	1.5
7	3	12
0.35±0.15	0.34±0.06	0.31±0.11
2.36±1.19	3.67±0.94	2.39±1.05
25.5±12.7	41.8±19.6	35.4±19.2
0.24±0.38	0.96±0.18	1.04±0.78
0.78±0.51	4.26±1.60	2.83±1.96
8.62±3.57	10.1±2.29	12.4±6.48
357±265	578±311	510±354
315±327	592±304	ND
6.49	10.5	ND
99.0	53.9	69.4
89.8±4.94	91.3±3.22	92.7±4.15

Abbreviations: *n*, number of patients; t_{lag}, lag time; t_{max}, time to peak plasma levels; C_{max}, maximum plasma concentrations; $t_{1/2}(i)$, half-life of the *i*-th disposition phase; AUC, area under the plasma concentration *versus* time curve; CV, coefficient of variation.

myelotoxicity grade according to common toxicity criteria (CTC) in the entire patient population correlated with the AUC of 9-AC lactone (r=0.93; p<0.001; not shown). The development of any myelotoxicity grade 2 or worse was associated with an AUC of 9-AC lactone ≥17.3 ng*h/mL, using the Hill equation and data shown in Fig. 3*B*. Pharmacokinetic/pharmacodynamic relationships based on (log-)linear and non-sigmoidal E_{max} models were less predictive, as were models based on 9-AC carboxylate or 9-AC total (not shown).

DISCUSSION

Topoisomerase I inhibitors are of great clinical interest because of their unique mode of action, their important antitumor activity and the high expression of the enzyme in various human tumor types. 9-AC, a semisynthetic analog of camptothecin revealed a broad antitumor activity in preclinical studies. Initial Phase I studies focused on schedules with prolonged infusion duration. In order to facilitate the prolonged drug administration, an oral formulation was developed. 9-AC can be administered orally as a colloid dispersion (CD) or as gelatine capsules in PEG1000. In dogs the mean oral bioavailability of the CD formulation was 13% (range, 4.5-26%), as compared to 10% of

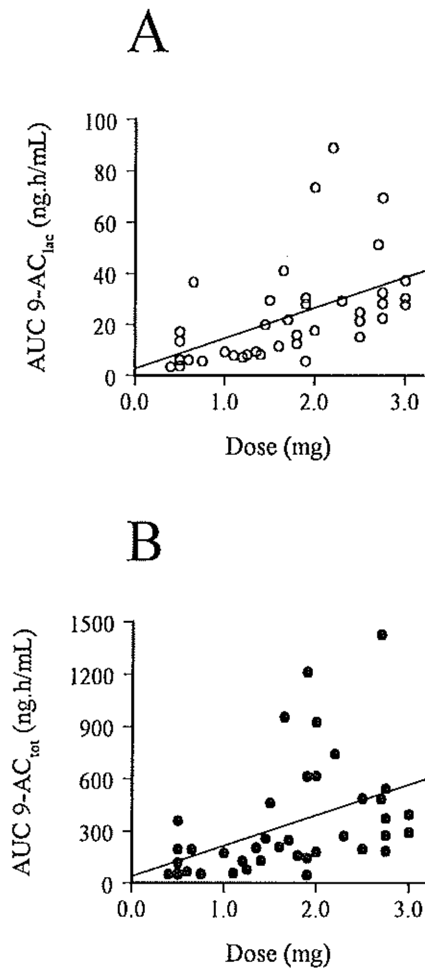

Fig. 3: Absolute dose of 9-AC plotted versus AUC of 9-AC lactone (A) and 9-AC total (B).

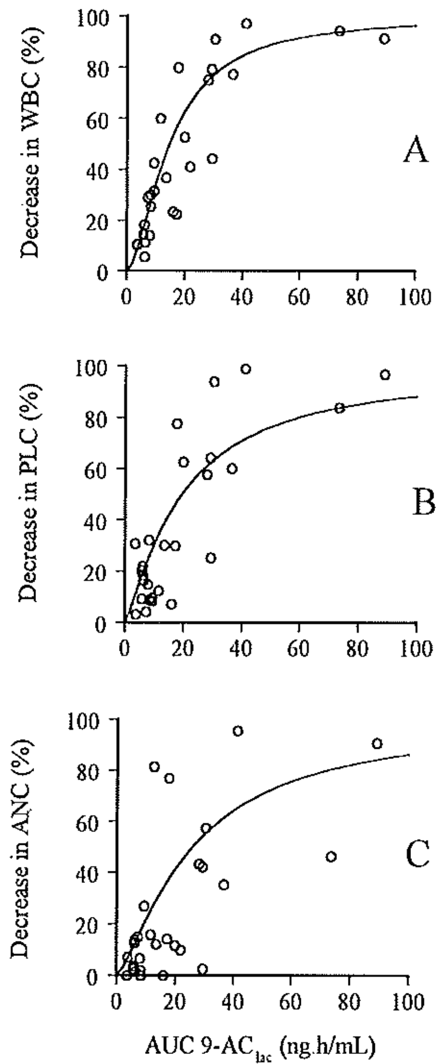

Fig. 4: Correlation between the area under the plasma concentration-time curve (AUC) of 9-AC lactone and the percentage decrease in white blood cells (WBC) at nadir of the first treatment course (A), in platelets (PLC) (B) and in neutrophiles (ANC) (C). The lines represent the fitting of the data to a sigmoidal maximum-effect model.

the PEG1000. Both formulations retained their antitumor activity after oral administration. Recently the phase I study on the oral administration of the CD formulation of 9-AC, 5 days per week, every 2 weeks was completed [25]. Diarrhea was the dose limiting toxicity at a dose level of 0.2 mg/m^2. PEG1000 9-AC was previously shown to demonstrate rapid intestinal absorption in patients after oral delivery, with an overall bioavailability (F) of 48.6±17.6%. This compares favorable to other camptothecin analogs, including topotecan (F=30.0%) [26], 7-(4-methyl-piperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin (GI147211; F=11.3%) [27], and irinotecan (F=12-21%) [28]. The terminal half-life ($t_{1/2}$) of 9-AC was shown to be 12.6±4.20 hr, which is substantially longer than that of topotecan (range, 2.35-5.91 hr) [26] and GI147211 (range, 6.85±3.13 hour) [27]. Another difference between topoisomerase I inhibitors constitutes the AUC ratio of the active lactone and the total drug of the parent drug. The conversion of 9-AC lactone into the ring-opened species in plasma could be demonstrated from the first sample acquired (i.e. at 0.33 hr). At equilibrium, the 9-AC carboxylate accounted for 91.1±2.11% of 9-AC total drug concentrations This compares unfavorable to the percentages present in the ring-opened form at equilibrium observed in patients for topotecan (~50%) [26], GI147211 (~60%) [27], and irinotecan and its active metabolite SN-38 (~65% and ~35%, respectively) [29]. These figures underscore the important differences in pharmacokinetics between camptothecin analogs.

Our results of the Phase I study with oral 9-AC capsules indicated that the drug could be administered in a 14-day schedule repeated every 3 weeks with tolerable and manageable toxicity [30]. The dose-limiting toxicities were a combination of thrombocytopenia *plus* neutropenia complicated by fever and diarrhea occurring at a dose level of 1.1 mg/m^2/day. Other side effects were mild to moderate (CTC grade 1 to 2) and consisted of nausea, vomiting, alopecia, mucositis and fatigue. Although 9-AC demonstrated a linear pharmacokinetic behavior over the entire dose range studied, we observed that the AUC of 9-AC lactone was a better indicator for the observed hematological toxicity than the dose. The intrapatient variability in AUC and peak drug levels was extremely small and averaged less than 10% for 9-AC lactone. However, the interpatient variability in the concentrations of 9-AC at each of the sample-time points as well as in the AUC was large, with values for the coefficient of variation as high as 99%. In this study, the high variability in lactone to carboxylate interconversion was significantly related to individual differences in pretreatment serum albumin levels. Although our results need to be confirmed in a larger number of patients, they tend to indicate that higher protein levels will result in a more profound binding of 9-AC carboxylate, thereby further diminishing the effective concentration of the active species of the drug. In all, these data indicate that classical drug dosing based on body-surface area alone is unlikely to be effective in minimizing interpatient differences in systemic exposure to oral 9-AC.

The pharmacokinetics of 9-AC were clearly related to the pharmacodynamic outcome, (i.e. hematological toxicity). The sigmoidal E_{max} model was found most appropriate to fit the kinetic data to the observed myelosuppression. The best correlation was obtained with the AUC of 9-AC lactone,

the exposure to the active drug. Considering this pharmacokinetic-pharmacodynamic relationship, a target AUC for 9-AC lactone can be defined according to the grade of toxicity that is considered to be acceptable in future studies. If hematological toxicity graded 2 or less is defined as acceptable, then the target AUC of 9-AC lactone is 17.3 ng*h/mL, using the Hill equation and data shown in Fig 3*A*.

For pharmacokinetic and pharmacodynamic analysis frequent blood sampling is inevitable. In order to evaluate salivary drug monitoring of 9-AC as an alternative to drug monitoring in plasma, the concentrations and AUC of 9-AC total and lactone were measured in unstimulated saliva samples in 5 patients during this study. The 9-AC concentration ratio in plasma and unstimulated saliva proved to be strongly patient-dependent and highly variable, suggesting that saliva is an unreliable matrix for pharmacokinetic analysis of 9-AC. Similar results were obtained recently for pharmacokinetic analysis of CPT-11 and SN-38 in saliva demonstrating large interpatient variability in plasma/saliva ratios [31].

Recently, we developed a limited-sampling model for reliable and accurate prediction of the systemic exposure to 9-AC after oral drug administration, using only one time blood sample taken at 3 hr after drug dosing [32]. In order to further diminish the interpatient variability in drug exposure in future studies, a pharmacokinetic guided approach may be considered. After oral administration of an appropriate starting dose of 9-AC (i.e. 1 mg/m^2), the 9-AC lactone plasma concentration can then be measured at 3 hr after drug dosing. Using the limited-sampling model and the linear-regression relationship between drug dose and AUC (Fig. 3), the optimal dose leading to the target AUC, determined according to the toxicity considered acceptable, can be calculated.

This procedure may prove valuable in reducing interpatient variation in exposure to 9-AC, and will enable us to optimize the treatment for any given patient by combining maximally achievable doses with tolerable toxicity during treatment. This strategy seems to be interesting both in further phase II studies using the 14 day administration schedule and in Phase I studies with different schedules of administration.

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Chapter 4

Liposomal lurtotecan (NX 211)

Chapter 4a

Liposomal lurtotecan (NX 211): determination of total drug levels in human plasma and urine by reversed-phase high-performance liquid chromatography

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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

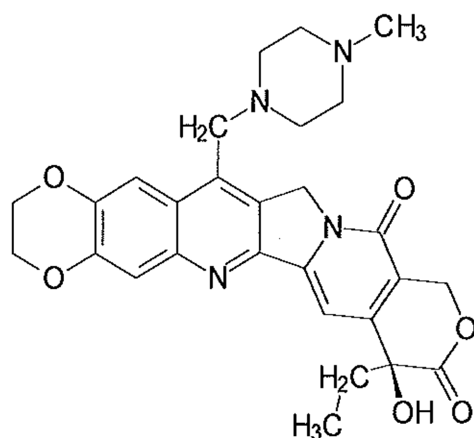

Fig. 1: Chemical structure of lurtotecan (LRT).

exposure to the pharmacologically active drug form. 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.

Sample treatment 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 23,000xg (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	✔	✔
Column oven SpH99	T=60°C	T=60°C
Column ODS-80A	✔	✔
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 4000xg (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,

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

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).

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. 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

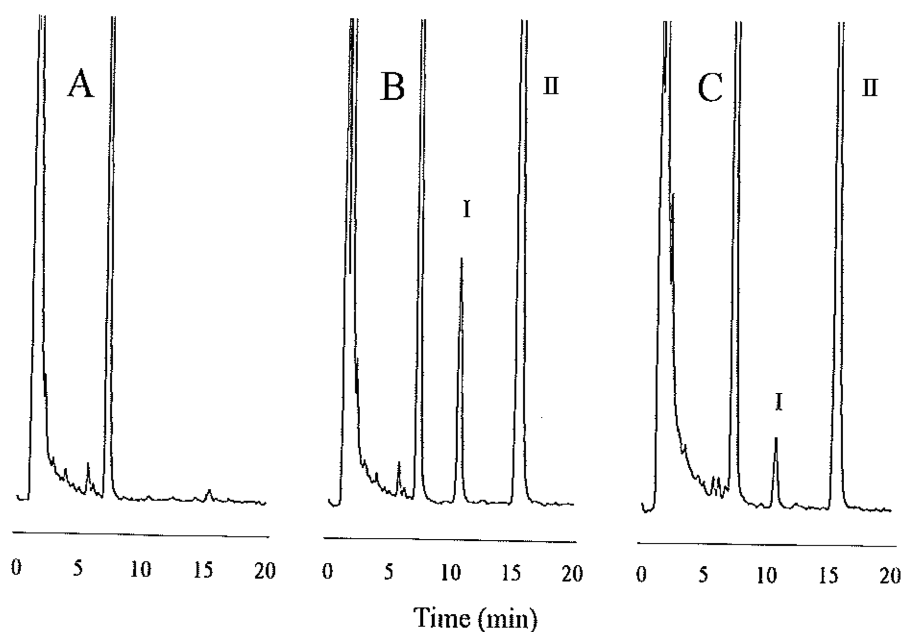

Fig. 2: Chromatograms of a blank human plasma sample (A), a plasma sample spiked with 10.0 ng/ml LRT free base (B) and a plasma sample obtained from a patient 8 h after the administration of NX 211 at a dose level of 0.8 mg (C). Peaks labeled I and II correspond to LRT and the IS, respectively.

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

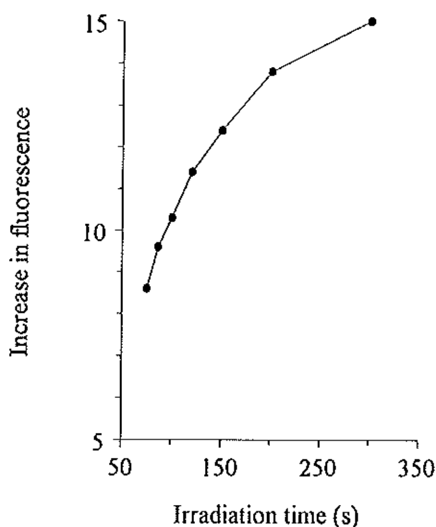

Fig. 3: Influence of the photochemical reaction unit on the fluorescence intensity of LRT.

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. 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.

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

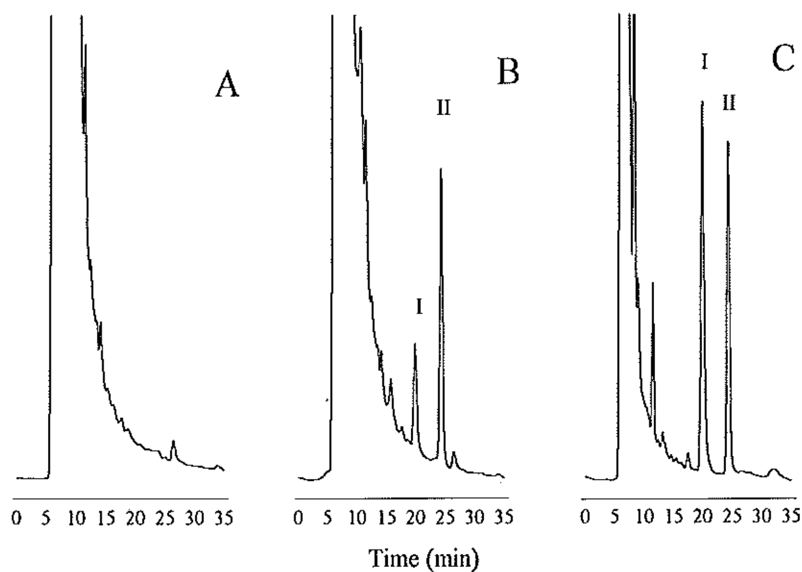

Fig. 4: Chromatograms of a blank human urine sample (A), a urine sample spiked with 2.50 ng/ml LRT free base (B) and a urine sample obtained from a patient, collected 12-24 h after the administration of NX 211 at a dose level of 0.8 mg (C). Peaks labeled I and II correspond to LRT and the IS, respectively.

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.

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.

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Chapter 4b

Liposome-encapsulation significantly reduces lurtotecan (NX 211) clearance in cancer patients

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ABSTRACT

This study was designed to evaluate the disposition of liposome-encapsulated lurtotecan (NX 211) in 6 cancer patients receiving the drug as a 30-min i.v. infusion (dose, 1.6–4.3 mg/m^2). Serial plasma, whole blood, urine, and feces samples were collected for 96 h and analyzed by reversed-phase high-performance liquid chromatographic assays. The pharmacokinetic profile in plasma was characterized by a slow systemic clearance of 0.78±0.88 L/h/m^2 (mean±SD) with a mono-exponential decline, and a steady-state volume of distribution approximating the blood volume (3.5±2.6 L/m^2). In addition, the total lurtotecan blood:plasma concentration ratios averaged 0.66±0.13, indicating limited drug accumulation in blood cells. Urinary excretion was 8.2±4.7% of the delivered dose, indicating that renal clearance is a minor route of elimination of the unchanged drug. Total fecal excretion amounted to 9.7±6.2% of the dose, and 2 potential metabolites could be identified from fecal extracts. Overall, these data indicate that NX 211 administration results in significantly increased plasma exposure as compared to non-liposomal lurtotecan, which may be a potential advantage with pharmacodynamic importance.

INTRODUCTION

Lurtotecan is a totally synthetic analog of 20-(*S*)-camptothecin, a natural product isolated from *Camptotheca acuminata* [1]. Structurally, lurtotecan is unique among camptothecin drugs, because of a dioxolane moiety on the A-ring and a bulky 4-methyl-piperazinomethylene group on the C-7 position (fig. 1). These molecular modifications have resulted in enhanced aqueous solubility as compared to the original agent and increased affinity of the compound for DNA topoisomerase I, the cellular locus through which camptothecin analogues produce their antitumor activity. The cytotoxicity of these so-called topoisomerase I inhibitors is distinctly S-phase specific, and various preclinical studies with lurtotecan as well as other camptothecin drugs have suggested that prolonged exposure, achieved either by repeated doses or prolonged infusion, might be beneficial for efficacy profiles (reviewed in [2]). In order to exploit this apparent schedule dependency, several clinical trials have been conducted with lurtotecan focussing on intermittent (daily for 5 days every 3 weeks [3-5]) or prolonged i.v. dosing schedules (72-h infusion every 4 weeks [6] or 7-, 14-, or 21-day infusions [7]). These studies have shown that the pharmacokinetic behaviour of lurtotecan is influenced significantly by a chemical, pH-dependent hydrolysis of the lactone functionality in the core structure, generating a ring-opened carboxylate form, which is devoid of biological activity [8]. Based on this finding, considerable effort has been put recently in the development of alternative pharmaceutical vehicles that would allow prolonged systemic exposure to the active lactone form.

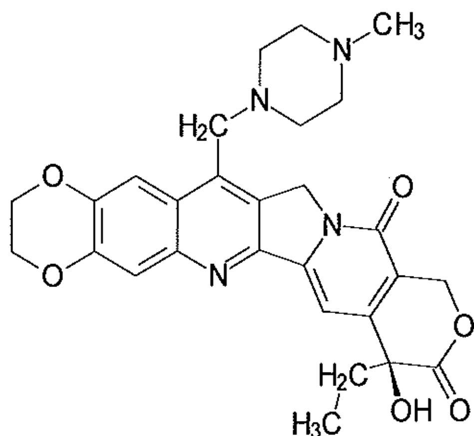

Fig. 1: Chemical structure of lurtotecan.

Among various approaches, liposomal encapsulation of camptothecin analogues was shown to be very efficient against lactone hydrolysis and, in fact, increased antitumor activity in mouse models by enhancement of tissue distribution and systemic drug availability [9-13]. Here, we have examined the disposition of a new liposomal formulation of lurtotecan (NX 211) in a group of cancer patients to investigate the clinical utility of this concept.

PATIENTS AND METHODS

Patients and treatment

Patients with a histologically confirmed diagnosis of advanced solid tumor refractory to standard therapy were eligible for the present study. Additional eligibility criteria included: age ≥18 years; Eastern Cooperative Oncology Group performance status ≤2; no previous treatment with antineoplastic agents for at least 3 weeks (4 weeks in case of carboplatin or any investigational agent and 6 weeks in case of nitrosureas or mitomycin C); adequate hematopoietic (absolute neutrophil count $>1.5\times10^9$/L, and platelet count $>100\times10^9$/L), renal (serum creatinine within normal limits), and hepatic function (total serum bilirubin within normal limits, and aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT)

levels ≤ 2.5 times upper normal limits); 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 Review Board, and all patients signed informed consent before study entry.

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 obtained from Gilead Sciences Inc. (San Dimas, CA, USA). The drug product was supplied in 50-mL vials containing 5 mg lurtotecan. Each vial contained 80 mg hydrogenated-soy phosphatidylcholine, 19 mg cholesterol, 0.9 g sucrose, 2 mg citric acid, 5 mg ammonium chloride to a total volume of 10 mL. Dose solutions for administration were prepared in polyvinyl chloride-free infusion containers protected from light and under aseptic conditions by dilution of the pharmaceutical preparation with sterile 5% dextrose (D5W) to a volume of 25 mL. The NX 211 dose (1.6, 3.2 or 4.3 mg/m^2) was administered as a 30-min i.v. infusion.

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 5-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 h after start of infusion. At each sampling time point, 1 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 min 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-h or 24-h portions, and aliquots were stored frozen in polypropylene vials. Complete collections of feces 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 (PBS) 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, USA) and was used as standard for all reversed-phase high-performance liquid chromatographic assays (HPLC). Plasma and urine concentrations of lurtotecan were determined using validated HPLC assays as described previously [14]. The sample pretreatment for the analysis of total plasma concentrations (i.e., the total of lactone plus carboxylate levels) consists of protein precipitation with 10% (w/v) aqueous perchloric acid-acetonitrile (2:1, v/v). Lurtotecan was separated from endogenous compounds on an Inertsil-ODS

80A column, with a mobile phase composed of 1 M aqueous ammonium acetate-water-acetonitrile (100:725:175, v/v/v). The flow rate was set at 1.25 mL/min, the temperature was maintained at 60°C and the column effluent was monitored with excitation and emission wavelengths of 378 and 420 nm, respectively. Urine concentrations of lurtotecan were quantified after a solvent extraction with *n*-butanol-diethyl ether (3:4, v/v), using the same column and mobile phase. The flow rate was set at 0.75 mL/min and the fluorescence signal of lurtotecan was increased 14-fold prior to detection by exposure of the effluent to UV light (254 nm) in a photochemical reactor unit. The LLQs were 1.00 and 0.50 ng/mL for the determination of total lurtotecan concentrations in human plasma and urine, respectively.

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), a Inertsil-ODS 80A analytical column (150x4.6 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 25m x 0.3mm 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 flow rates set at 1.25 and 0.75 mL/min for the determination of total lurtotecan levels in blood and fecal samples, respectively.

Aliquots of 50 μL 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 min on a multi-tube vortex mixer, followed by centrifugation for 5 min 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 PBS in 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 PBS to the whole blood. In addition, to minimize potential differences with clinical samples, a recovery control sample containing 7.50 ng/mL lurtotecan (as NX 211), was also analyzed simultaneously. The sample containing 2000 ng/mL was diluted 100-fold in PBS prior to extraction.

Aliquots of 100 μL feces homogenates were deproteinized and acidified with 1000 μL of 5% (w/v) aqueous perchloric acid-acetonitrile (5:1, v/v) containing 100 ng/mL 6,7-dimethoxy-4-methylcoumarin (Sigma, St. Louis, MO, USA), which was used as the internal standard.

Subsequently, the samples were vigorously vortex-mixed for 15 min on a multi-tube vortex mixer, followed by centrifugation at ambient temperature at 23,000xg for 5 min. A volume of 100 μL 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 PBS from the lurtotecan working solution to 240 μL drug-free feces homogenates. Three pools of quality-control samples containing 40, 200 and 2000 ng/mL lurtotecan were prepared by addition of appropriate volumes of lurtotecan in PBS to blank human feces homogenates. The sample containing 2000 ng/mL was diluted 10-fold in a mixture of PBS 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 lower limit of quantitation and quality-control samples in quintuplicate, and was performed on 4 separate occasions. The lower limit of quantitation samples were prepared daily in drug-free heparinized whole blood samples and fecal homogenates of 5 healthy volunteers. Within-run and between-run precisions were calculated by one-way ANOVA for each concentration using the run-day as variable. The extraction recoveries for lurtotecan and the internal standard in the assay for lurtotecan in feces specimens were calculated by comparing peak heights obtained from an extracted sample containing 100 ng/mL lurtotecan in PBS to those obtained in extracted calibration samples. The extraction recoveries for lurtotecan in the assay in whole blood were calculated using the data of the quality and recovery-control samples in comparison to calibration standards.

Table 1: Characteristics of the studied patients

Patient No.	Dose (mg/m^2)	Gender	Age (yrs)	Tumor type	BSA (m^2)	Ht (L/L)
1	1.6	Female	61	NSCLC[a]	1.63	0.30
2	1.6	Female	74	sarcoma	1.56	0.28
3	3.2	Female	57	myosarcoma	2.09	0.40
4	3.2	Female	40	SCLC	2.06	0.37
5	3.2	Male	52	bile duct carcinoma	1.88	0.41
6	4.3	Male	55	bladder carcinoma	1.98	0.36

a: NSCLC, non-small cell lung cancer; SCLC, small-cell lung cancer; BSA, body-surface area; Ht, hematocrit on day 1

Pharmacokinetic analysis

Individual plasma and whole blood concentrations of lurtotecan were fit to a model with multi-exponential functions using the Siphar version 4.0 software package (SIMED, Créteil, France), using Powell's method. 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} (not shown). The disposition half-life was calculated as $ln2/k$, 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 AUC. The volume of distribution at steady state was calculated using the same program.

RESULTS

Complete pharmacokinetic studies were performed in 6 patients entered onto a phase I clinical trial of liposomal lurtotecan (NX 211) given as a 30-min i.v. infusion [15]. Full clinical toxicities and treatment responses will be reported in detail elsewhere in due course. The group consisted of 4 females and 2 males ranging in age from 40 to 74 years (Table 1). The median clinical chemistry values for these patients included total bilirubin levels of 6 µM (range 5-11), serum creatinine levels of 90 µM (range 75-108), ASAT and ALAT of 27 units/L (range 19-47) and 20 units/L (range 8-57 units/L), respectively.

Analytical methods

The lurtotecan plasma concentration values for patients treated with NX 211 reported here are the sum of both encapsulated and non-encapsulated drug. In order to gain a preliminary insight into the disposition and elimination of NX 211 in humans, the assay procedures as described recently [14] were slightly modified for the determination of total lurtotecan concentrations in human whole blood and fecal homogenates. PBS was used as matrix for the construction of the calibration standards for the determination of total lurtotecan levels in human heparinized whole blood, since pools of drug-free heparinized whole blood were not available. Because of this, we focussed on an extraction procedure with quantitative (i.e., 100%) recovery. Eventually, the extraction recoveries for lurtotecan, using the described method, were 103, 100 and 97% for the quality-control samples containing 1.25, 20.0 and 2000 ng/mL of lurtotecan and 101% for the recovery-control sample containing 7.50 ng/mL of NX 211. However, the extraction recovery for the internal standard still did not reach 100% (data not shown), and, hence, no internal standard was used in this assay. The calibration curves were linear over the entire range studied, with the validation characteristics of the quality control

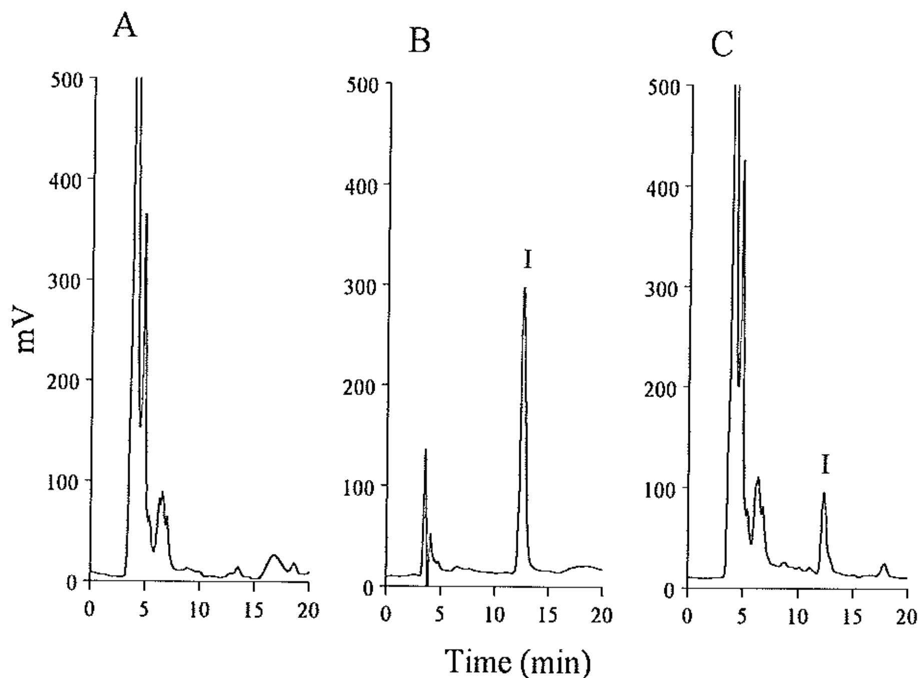

Figure 2: Chromatograms of whole blood samples of patient 6; blank (A), a 100-fold diluted sample obtained at the end of the infusion containing 1265 ng/mL lurtotecan (B) and a sample taken 96 h after the start of the infusion containing 3.7 ng/mL lurtotecan (C). Peak labeled I correspond to lurtotecan.

samples summarized in Table 2. Fig. 2 shows representative chromatograms of lurtotecan in whole blood samples of patient 6 after the administration of NX 211. Similar data were obtained for the determination of lurtotecan in feces homogenates (Table 2), with recoveries of lurtotecan and the internal standard of 100 and 98.5%, respectively. Representative chromatograms of lurtotecan in fecal extracts of the same patient are shown in fig 3.

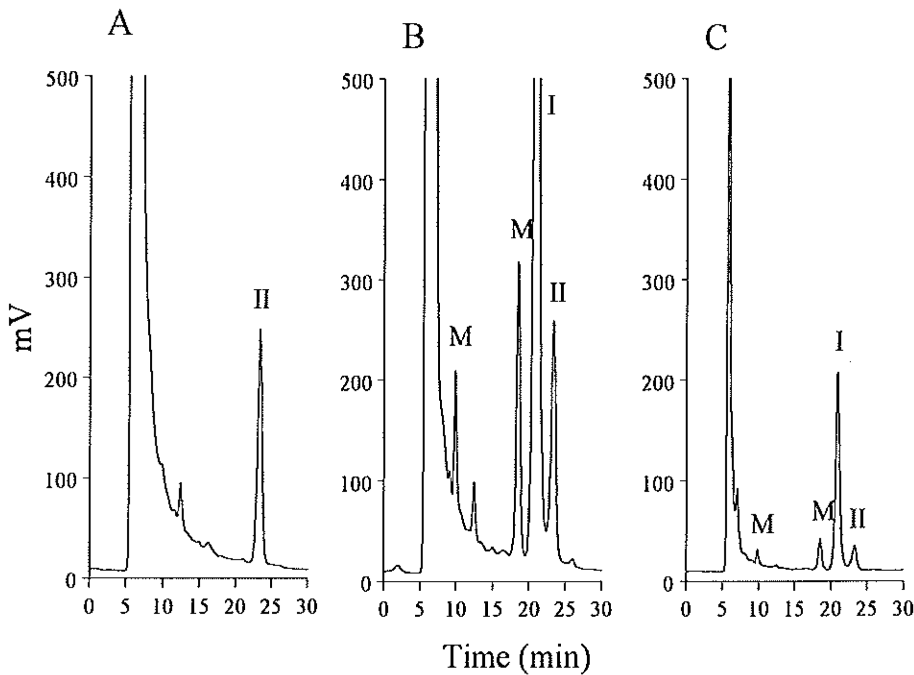

Figure 3: Chromatograms of fecal extracts of patient 6: blank (A), an undiluted fecal homogenate from a feces sample collected at 54 h after the start of the infusion with 2 potential metabolites (B) and the same sample 10-fold diluted after extraction containing 976 ng/mL lurtotecan (C). Peaks labeled I, II and M correspond to lurtotecan, internal standard and potential metabolites, respectively.

Disposition of lurtotecan

In table 3, the pharmacokinetic data are summarized of total lurtotecan, which could best be fitted with a mono-exponential decline, in plasma as well as in whole blood. Characteristic were the slow plasma clearances of the drug of 0.10 – 2.38 L/h/m^2, the mono-exponential decline with a half life of 2.42 – 11.2 h and the low steady state volume of distribution, ranging from 1.58 – 8.31 L/m^2. The mean blood:plasma total lurtotecan concentration ratio of 0.66±0.13 is

indicative for liposomal encapsulation of lurtotecan in the plasma compartment, since no or limited drug is accumulated into the red blood cells (fig 4). Figure 5 shows the kinetic profile of total lurtotecan of patient 6 in plasma and whole blood. An additional chromatographic peak was found in the plasma samples with a retention time of approximately 50 minutes (not shown), which had an equal retention time as the chromatographic peak in the recovery control samples as described recently [14]. Structural identification as well as pharmacologic properties of this compound will be discussed in a separate communication shortly.

The urinary and fecal excretions of the unchanged parent compound lurtotecan are listed in table 3. The total excretion of lurtotecan in urine and feces ranged from 8.5 – 38% in the 6 studied patients, suggesting extensive metabolism of lurtotecan. Inspection of the HPLC chromatograms from fecal extracts revealed 2 major additional peaks, which were absent in the blank fecal extracts, that might represent lurtotecan metabolites (fig 3). In fig 6, the cumulative excretions of lurtotecan in urine and feces of patient 6 are shown.

Table 2: Validation characteristics of quality-control samples for the two HPLC assays

Nominal (ng/mL)	Observed (ng/mL)	WRP[a] (%)	BRP (%)	ACC (%)
Lurtotecan in whole blood				
0.25[b]	0.24	7.8	12.4	94.9
1.25	1.29	11.4	5.1	103
20.0	20.2	8.7	7.5	101
2000	1950	13.2	c	98.0
Lurtotecan in feces homogenate				
10[b]	11	5.0	3.9	106
40	41	4.3	6.9	101
200	203	2.9	6.7	101
2000	1926	3.6	8.6	96.3

a: 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.

DISCUSSION

In the present study, we have described for the first time the human pharmacokinetics of a liposome-encapsulated camptothecin analogue in plasma, whole blood, urine and feces.

Of the greatest importance for the antitumor activity of lurtotecan treatment is the disposition of the agent in plasma. Lurtotecan has shown to be an active compound in animal models as well as in clinical trials. In general, for topoisomerase I inhibitors, prolonged exposure to the agent has been associated with an increase in cytotoxicity. Of particular note, the phase I data for free lurtotecan suggest that antitumor activity may be enhanced with continuous infusion, since responses were observed in the 72-h and 21-day continuous infusion schedules [6,7] As far as toxicity is concerned, although prolonged infusion is associated with an increase in thrombocytopenia, it has not been associated with an increase in the incidence and severity of neutropenia. Therefore, the use of a liposomal formulation of lurtotecan, as administered here, may also improve efficacy by increasing exposure to the active (lactone) drug form and modifying the safety profile, thus enhancing the therapeutic index of the parent compound.

Table 3: Pharmacokinetic parameters of lurtotecan after NX 211 administration

Patient No.	CL[a] ($L/h/m^2$)	$V_{d,ss}$ (L/m^2)	$T_{1/2}$ (h)	Fe_u (%)	Fe_f (%)	Ratio bl/pl
1	2.38	8.31	2.65	5.4	9.1	0.80
2	0.67	3.94	3.99	4.9	3.6	0.70
3	0.10	1.58	11.2	8.2	6.4	0.54
4	0.28	1.76	4.18	6.1[b]	6.2	0.81
5	1.15	3.63	2.42	7.3	11.7	0.57
6	0.11	1.65	10.6	17.4	21.0	0.52
mean	0.78	3.48	5.84	8.6	9.7	0.66
SD	0.88	2.59	3.99	5.1	6.2	0.13
CV	113%	74%	68%	59%	64%	20%

a: CL, total plasma clearance; MRT, mean residence time; $V_{d,ss}$, volume of distribution at steady-state; $T_{1/2}$, half-life of the terminal disposition phase; Fe_u, fraction of the delivered dose excreted as unchanged drug in urine within 96 h; Fe_f, fraction of the delivered dose excreted as unchanged drug in feces within 96 h; %CV, coefficient of variation. b: Incomplete recovery (0-12 h urine sample missing), not used for calculations.

Here, we have shown that the disappearance of lurtotecan after NX 211 administration was characterized by a mono-exponential decline with a terminal disposition half-life in plasma of approximately 6 h. This is in contrast to the multiphasic elimination from plasma demonstrated by lurtotecan, which displays a terminal half-life, estimated as 9.6±4.8 h in a cohort of 14 patients [4]. 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 possible resulting in the same terminal half-life as lurtotecan, but with concentrations below the lower limit of quantitation of the assay. The total lurtotecan plasma clearance from NX 211, on average 0.78 L/h/m^2, is 25 times slower than the clearance of the free drug, which was established at 21±9.6 L/h/m^2 [4]. The observed steady

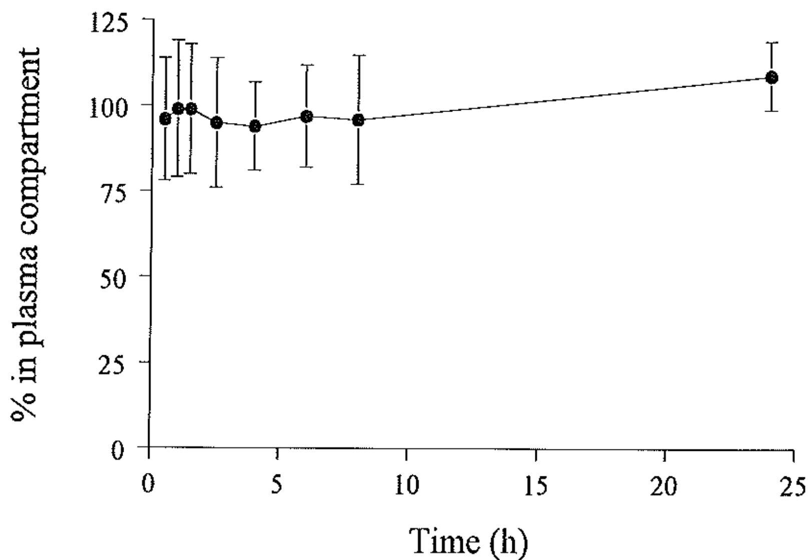

Figure 4: Disposition of lurtotecan in the blood compartment in the studied patients (mean ± S.D.).

state volume of distribution of 3.48±2.59 L/m^2 and the blood/plasma ratio of 0.66±0.13 are indicative for encapsulation of lurtotecan in the liposomes, which are mainly located in the plasma compartment.

The observed variability in the pharmacokinetic behavior of lurtotecan after the administration of NX 211 is slightly higher to that reported for the free drug, with an interpatient variability in the plasma clearance of 113% for NX 211, versus 46% [4] for free lurtotecan, while these values for the volume of distribution were 74% and 52% [4], respectively.

Urinary excretion of the unchanged drug was 8.6 ± 5.1% in the 6 studied patients, which is similar with earlier findings of 14.3 ± 7.0% [3] and 11% [4] after a 30 min infusion of free drug, indicating that renal clearance plays also a minor role in the elimination of NX 211. Since also the total amount

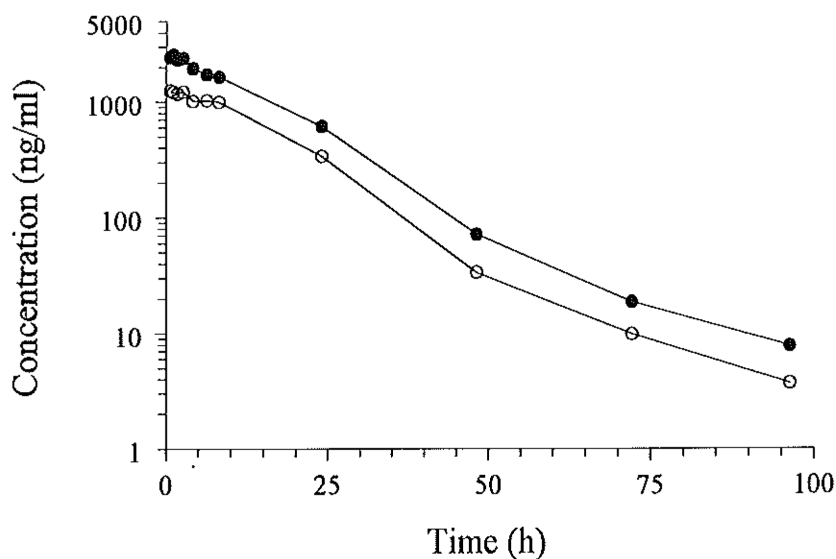

Figure 5: Kinetic profile of lurtotecan in plasma (closed circles) and whole blood (open circles) in patient 6 after administration of 8.5 mg NX211.

of lurtotecan in feces accounted for only 9.7% of the administered dose, lurtotecan is probably extensively metabolized. At least 2 potential metabolites of lurtotecan were observed in the chromatograms of fecal homogenates, which will be part of further research regarding the metabolism of NX 211.

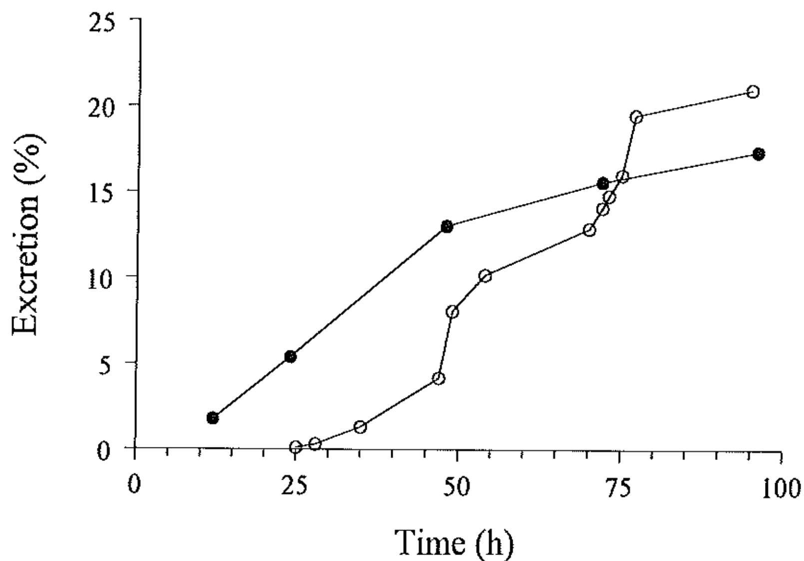

Figure 6: Cumulative excretion of unchanged drug in urine (open circles) and feces (closed circles) of patient 6 after administration of 8.5 mg NX211.

In conclusion, administration of liposome-encapsulated lurtotecan significantly reduces the plasma clearance of the drug, which in turn might prove beneficial for pharmacodynamic outcome. Toxicity and anti-tumor response in relation to the pharmacokinetics are currently under investigation in a clinical phase I trial.

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Summary and conclusions

Over the last decade, several analogues of the topoisomerase I inhibitor camptothecin, including topotecan, irinotecan, lurtotecan and 9-aminocamptothecin, have been introduced into clinical practice. Topoisomerase I is a nuclear enzyme involved in the replication of DNA, by forming a covalent binding with DNA, the cleavable complex. This cleavable complex is stabilized by these camptothecins, resulting in a single strand breakage of the DNA, which finally leads to cell death.

The camptothecin analogues share a pH-dependent reversible conversion between the pharmacologically active ring-closed lactone form and its inactive, lactone ring-opened, carboxylate form. A quantitative measurement of these analogues is rather complex because of the chemical instability of the lactone moieties of the camptothecin analogues.

Methodologies for the quantitative determination of the topoisomerase I inhibitors topotecan, 9-aminocamptothecin and lurtotecan are described and the applicability of these assays is shown in this thesis.

In **Chapter 1**, an overview is given of the methodologies currently used for the determination of topotecan, irinotecan, 9-aminocamptothecin and lurtotecan. Since these camptothecins have strong fluorescence characteristics, the analytes were quantified by fluorescence detection. As mentioned, the camptothecin analogues have a pH-dependent reversible conversion between the pharmacologically active lactone form and the inactive carboxylate form, which necessitates rapid centrifugation of the blood samples to collect the plasma. Also when only total concentrations, i.e. lactone plus carboxylate, are to be measured, this direct blood processing is crucial, since the lactone form is able to diffuse across the cell membranes of red blood cells, and thus a change in the lactone to carboxylate ratio has an effect on the total drug concentrations in the plasma compartment. Stabilization of the lactone to carboxylate ratio by direct freezing of the plasma sample is the most convenient approach for the determination of the lactone only concentrations. On the day of analysis all samples can be handled at once by solid-phase or liquid-liquid extraction techniques, in which only the lipophilic lactone form is extracted, while the carboxylate form is discarded. The total drug concentrations are measured in a second analysis after acidification of the samples. Another, simple way of stabilizing the lactone to carboxylate ratio is by cold methanolic deproteinization of plasma samples, immediately after collection of the plasma. The lactone and carboxylate concentrations are measured simultaneously in one analytical run. However, up to now, this approach has only been shown to be feasible for the determination of the two forms of topotecan and irinotecan.

Pharmaceutical and biomedical aspects of topotecan, a semisynthetic water-soluble camptothecin analogue, are described in **chapter 2**. The intravenous formulation of topotecan (Hycamtin®) has been registered for the treatment of ovarian cancer in Europe and the USA,

while an oral formulation is still under clinical investigation.

In **chapter 2a** the simultaneous determination of the lactone and carboxylate forms of topotecan in human plasma and of the total concentration in human urine is described. The plasma clean up is based on simple methanolic protein precipitation, which stabilizes the lactone to carboxylate ratio when stored at –80°C. The lower limit of quantitation for the lactone as well the carboxylate form is established at 0.10 ng/ml, with an overall run time of 10 min, which enables analysis of complete runs of patient samples during day time. Total topotecan concentrations in human urine samples, with a lower limit of quantitation of 10 ng/ml, are measured in the form of topotecan lactone, after acidification with orthophosphoric acid.

The influence of third spaces, such as pleural- and ascitic fluid, on the pharmacokinetics of topotecan and the penetration of topotecan into these fluids is described in **chapter 2b**, in 4 patients in the presence and absence of third space volumes. The apparent topotecan clearance, the terminal disposition half-lives and the AUC ratios of lactone to total drug in plasma remained unchanged within the same patient in the presence or absence of pleural or ascitic fluid. The penetration of topotecan into the pleural and ascitic fluid showed a mean lag time of 1.61 h and ratios with plasma concentration increased with time after dosing in all patients, with a mean ratio of third space topotecan total drug AUC to that in plasma of 0.55. So, topotecan can be safely administered to patients with pleural effusions or ascites, with substantial penetration of topotecan into these third spaces for local antitumor effects.

In **chapter 2c**, the pharmacokinetic interactions, and sequence dependent effects of orally administered topotecan in combination with i.v. cisplatin were studied in a phase I and pharmacological study. Cisplatin was given to a total of 49 patients at a fixed dose of 75 mg/m^2 as a 3-hour infusion, before topotecan on day 1 or after topotecan on day 5, in combination with oral topotecan at dose levels of 0.75 to 2.3 mg/m^2 for 5 days, repeated every 3 weeks. When topotecan was preceded by cisplatin, a more severe myelosuppression was induced, compared to the alternate sequence. The MTD for the most toxic sequence, cisplatin followed by topotecan, was established at a dose of 1.25 mg/m^2/dx5 of topotecan, while for the reversed sequence the MTD was found at a dose of 2.0 mg/m^2/dx5 of topotecan. The possible effect of drug sequence on the pharmacokinetics of topotecan and cisplatin was investigated in the first 18 patients, which were randomized in a crossover design for the administration sequence. No significant differences in the topotecan pharmacokinetics were found when topotecan was preceded or followed by cisplatin. As for topotecan, the pharmacokinetics of cisplatin were not influenced by the administration sequence. The antitumor efficacy at the MTD of both schedules should be evaluated in a randomized phase II study.

In the clinical practice of medical oncology, most anti-cancer drugs are commonly dosed based on the body-surface area of the individual patient, with the aim reducing inter-patient variability in drug exposure, which is based on an assumed relationship between the clearance of a compound and

the body-surface area of the individual patient. In **chapter 2d**, the relevance of body-surface area based dosing of orally administered topotecan in adult cancer patients is evaluated, by estimation of the intra- and inter-patient variabilities in topotecan lactone pharmacokinetics. The intra-patient variability in the topotecan lactone AUC, using the data of 47 patients who were sampled for 3 - 6 days, was 24 ± 13% (median 20%) with a range of 7.6 - 61%. The inter-patient variabilities in the apparent clearance of topotecan lactone, expressed in $L/h/m^2$ as well as in L/h, were 38 and 42%, respectively. In view of the relatively high intra- and inter-patient variabilities in the AUC and apparent clearance of topotecan lactone and the relatively small range in observed body-surface area of 12%, oral topotecan can be added to the list of agents where body-surface area adjusted dosing does not appear definitely better. Moreover, accurate dosing of oral topotecan based on body-surface area is also not feasible, since for oral use the drug is now only available as gelatine capsules containing 1.0 and 0.25 mg respectively, resulting in the necessity of rounding of the absolute dose to the nearest quartile mg. We recommend a fixed dose regimen for future use in clinical trials, which is more convenient for the oncologist and the pharmacist, is more cost-effective and last but not least, a fixed dose regimen is less cumbersome for the patients. Further randomized clinical studies are needed to fully explore the advantages of fixed dose regimens over body-surface area based dosing of orally administered topotecan in adult patients.

In **chapter 2e**, the gender-dependent differences in topotecan pharmacokinetics in adult cancer patients after oral as well i.v. administration and the explanation of this phenomenon is described. A significant 1.4-fold faster apparent clearance of topotecan lactone was found in males as compared to females treated with oral topotecan. After correction for the body-surface area, the apparent topotecan lactone clearance remains significantly 1.3-fold faster in males. In addition, a significant 1.3-fold higher lactone to total ratio of the AUC in females was found. Linear regression analysis between the significantly different biochemical characteristics body-surface area and hematocrit and the apparent clearance of topotecan lactone, expressed in $L/h/m^2$, resulted in a significant correlation of the apparent clearance with the hematocrit but not with the body-surface area. As in the oral study, female patients enrolled in a continuous i.v. study also had a higher lactone to total steady state plasma concentration ratio. In *in vitro* studies with altered hematocrit values, a strong significant correlation was found between the hematocrit value and the lactone to total concentrations. In addition, the lactone to total plasma concentration ratio, in *in vitro* experiments, was significantly higher in blood of female volunteers as compared to male volunteers, with significantly higher topotecan lactone concentrations in the plasma compartment of the blood samples of the female volunteers. The implications of this gender-related difference in topotecan pharmacokinetics on the pharmacodynamics and tumor responses have to be evaluated in a large study in which topotecan is administered at a fixed dose.

Chapter 2f is describing a clinical phase I study with increased oral topotecan dosages, in comparison with the dosages used in chapter 2c, in combination with a lower fixed dose of 50 mg/m^2 of i.v. administered cisplatin. The dose-limiting toxicities consisted of myelosuppression and gastrointestinal toxicity at a topotecan dose-level of 1.75 $mg/m^2/d$ administered on day 1-5, preceded by 50 mg/m^2 cisplatin on day 1. The pharmacokinetics of topotecan and cisplatin were similar to earlier data. The MTD of i.v. cisplatin at a dose-level of 50 mg/m^2 on day 1, directly followed by 5 days of oral topotecan at a dose of 1.50 mg/m^2, has no advantage as compared to our previous recommended schedule for phase II studies of i.v. cisplatin at a dose of 75 mg/m^2 on day 1 in combination with 1.25 $mg/m^2/d$ for 5 days of oral topotecan (chapter 2c), since only a minor dose escalation of topotecan could be achieved by a dose-reduction of 33% of cisplatin.

The poor water-soluble synthetic camptothecin derivative 9-aminocamptothecin, which has shown promising anti-tumor efficacy in xenograft models, is discussed in **chapter 3**. Due to the water solubility problems, 9-aminocamptothecin seemed to be inappropriate for further clinical development. Since a colloidal dispersion formulation has been developed, phase I and II clinical trials with i.v. administered 9-aminocamptothecin have been conducted, while gelatin capsules were developed for oral administration.

In **chapter 3a**, two high-performance liquid chromatographic methods for the determination of the lactone and total (i.e. lactone plus carboxylate) 9-aminocamptothecin concentrations in human plasma are described, with lower limits of quantitation of 50 and 100 pg/ml, respectively. The lactone form of 9-aminocamptothecin was extracted from the plasma by a single liquid-liquid extraction. The sample preparation for the determination of total plasma levels consists of a simple deproteinization/acidification step, in which the carboxylate form is converted to the lactone form. Both assays are sensitive, selective, accurate and reproducible and have been implemented in several clinical and *in vitro* studies.

The factors responsible for the phenomenon of rebound concentrations in the kinetic profile of total 9-aminocamptotheicn after a bolus i.v. infusion, at approximately 2-3 hours after dosing, are described in **chapter 3b**. After a 5-min bolus infusion, 9-aminocamptothecin is rapidly hydrolyzed, with less than 10% present in the pharmacologically active lactone form at 3 hours after dosing. In *in vitro* experiments, in which 9-aminocamptothecin was incubated in fresh human heparinized whole blood, the lactone instantaneously accumulated in the red blood cells, resulting in low plasma concentrations of 9-aminocamptothecin. Subsequently, the lactone form is hydrolyzed to the ring-opened carboxylate form, which is not able to pass cell membranes. So in time, higher concentrations of total 9-aminocamptothecin were detected in the plasma compartment. In addition, the carboxylate form of 9-aminocampthothecin has a high affinity for human serum albumin, resulting in a shift of the equilibrium towards the carboxylate form, which in turn is responsible for the rebound concentrations of total 9-aminocamptothecin in the

plasma compartment of cancer patients, after a i.v. bolus infusion. The absence of the rebound peak in preclinical murine xenograft models is most likely related to differences in the lactone to carboxylate ratios compared to humans, with percentiles of 9-aminocamptothecin present in the lactone form at equilibrium in solutions of human or murine serum albumin of respectively 0.63 and 35.0 %.

In **chapter 3c** the clinical pharmacokinetics of 9-aminocampthotecin in adult cancer patients are described in plasma and saliva after oral administration, and relationships have been made with the pharmacodynamics. In contrast to other camptothecin derivatives, such as topotecan, lurtotecan and irinotecan, the conversion of the lactone form into the carboxylate form appeared to be much faster, with the equilibrium favoring the carboxylate form. The pharmacokinetics were linear and dose-independent and the intra-patient variability in lactone AUCs were extremely low, while the inter-patient variability in the lactone AUCs were high. The 9-aminocampothecin lactone concentration ratio in plasma and unstimulated saliva were patient dependent and highly variable, indicating that unstimulated saliva is an unreliable matrix for pharmacokinetic analysis of 9-aminocamptothecin. From one patient, also pleural samples were collected and analyzed for 9-aminocamptothecin. As for topotecan (described in chapter 2b), pleural effusions are not a major compartment for 9-aminocamptothecin distribution. The AUC of the pharmacologically active lactone form significantly correlates with hematological toxicities like percentage decrease in total white blood cells, platelets and neutrophils. Since the intra-patient variability in drug exposure was very small, 9-aminocamptothecin could be individually dosed, using a target AUC as endpoint, in future clinical trials.

In **chapter 4**, pharmaceutical and biomedical aspects of lurtotecan, a semisynthetic camptothecin analogue are described. In order to improve the efficacy of lurtotecan in cancer patients, the drug was encapsulated in liposomes.

Chapter 4a, is describing reversed-phase fluorescence high-performance liquid chromatographic methods for the determination of total lurtotecan concentrations in human plasma and urine in samples of patients after administration of NX 211 (i.e. a liposomal formulation of lurtotecan). For the determination of total plasma concentrations, the sample pretreatment consists of a simple deproteinization/acidification step, resulting in a lower limit of quantitation of 1.00 ng/ml. This method has also been validated for the analysis of lurtotecan in urine samples, in which the urine was diluted 40-fold in blank human plasma, with the lower limit of quantitation established at 100 ng/ml urine. Lurtotecan concentrations in urine <100 ng/ml were determined by a modified procedure, using a single solvent extraction technique. The fluorescence signal of lurtotecan was increased prior to detection by post-column exposure to UV light (254 nm) in a photochemical reaction unit, in which the piperazinomethylene moiety on C7 of the molecule is lost, resulting in a 14-fold higher fluorescence signal compared to the parent

compound. The LLQ of this assay was 0.500 ng/ml. The methods meet the current requirements as to validation of bioanalytical methodologies and are implemented in an ongoing clinical phase I trial of NX 211.

The clinical applications of the assays described in chapter 4a, are described in **chapter 4b**, in which preliminary pharmacokinetics are shown of lurtotecan in patients treated with NX 211. In addition to the described assays in chapter 4a, in this chapter also assays and pharmacokinetics of lurtotecan in human whole blood and feces are described. As in the assay for the determination of total lurtotecan plasma concentrations, the sample treatment procedures of whole blood and feces samples consist of a simple protein precipitation/acidification step. In both assays, the photo-chemical reactor unit was used to increase the fluorescence signal of lurtotecan, resulting in lower limits of quantitation of 0.25 and 10 ng/ml in whole blood and feces, respectively. The pharmacokinetic profile in plasma was characterized by a clearance of 0.78 L/h/m^2, which is 25 times slower than these for the free drug, with a mono-exponential decline with a half-life of approximately 6 h, and a steady-state volume of distribution of approximately the blood volume. The low volume of distribution and the observed blood/plasma ratio of 0.66 are indicative for encapsulation of the drug in liposomes, which are mainly located in the plasma compartment. Urinary and fecal excretions of the parent compound lurtotecan were low, with mean values of respectively 8.6% and 9.7%, indicating that lurtotecan in probably extensively metabolized. In the chromatograms of the fecal homogenates 2 potential metabolites of lurtotecan were observed. The significantly reduced plasma clearance of lurtotecan by encapsulation of the drug in liposomes, might be beneficial for pharmacodynamic outcome. Toxicity and anti-tumor response in relation to the pharmacokinetics are currently under investigation in a clinical phase I trial.

Conclusions and future perspectives

The camptothecins belong to the class of topoisomerase I inhibitors and have shown antitumor activity against a broad range of human malignancies, including refractory ovarian and colorectal cancers. Over the last decades, several methodologies have been developed and validated for the determination of the lactone, carboxylate and total concentrations of camptothecin analogues in biological matrices. Since the lactone form is the pharmacological active form of these compounds, and the equilibrium between the lactone and carboxylate forms in plasma of the camptothecins differs from derivative to derivative and from species to species, the analysis of the lactone form is of great importance for pharmacokinetic-pharmacodynamic relationships.

In the future, the camptothecins will be increasingly combined with other drugs, because of which sensitive, selective, accurate and reproducible analytical methods for the determination of

camptothecin analogues, especially for the lactone forms of these drugs, are needed to get insight into possible pharmacokinetic interactions and their implications for the pharmacodynamics.

Generally, in the future, dosing of anti-cancer agents will hopefully be individualized, based on the pharmacokinetics of each individual patient, especially when more is known about pharmacogenetics, in order to get more efficient and less toxic therapies.

Samenvatting en conclusies

In de strijd tegen kanker worden steeds nieuwe methoden gezocht om de ongebreidelde groei van tumorcellen af te remmen. Eén van de nieuwste methoden is het blokkeren van het topoisomerase I enzym, dat betrokken is bij de deling van DNA en dus mede verantwoordelijk is voor de vermeerdering van cellen. DNA is een keten van nucleotiden dat in zich in een opgedraaide vorm in de celkern bevindt. Het topoisomerase I enzym rolt dit DNA af, waardoor andere enzymen voor de vermenigvuldiging kunnen zorgen, waarna het topoisomerase I enzym het DNA weer in zijn oorspronkelijke vorm terugbrengt. Door dit DNA-enzym complex te blokkeren, breekt het DNA, wat uiteindelijk resulteert in celdood. De laatste decennia zijn er diverse analoga van de topoisomerase I remmer camptothecine, zoals topotecan, irinotecan, lurtotecan en 9-aminocamptothecine ontwikkeld en getest in klinische studies.

Al de tot nu toe klinisch gebruikte derivaten van camptothecine hebben gemeen, dat er een zuurgraad afhankelijk evenwicht is tussen een farmacologisch werkzame lactonvorm en een niet werkzame carboxylaatvorm. Kwantitatieve bepalingen van deze topoisomerase I remmers worden door deze chemische instabiliteit van de lactonvorm bemoeilijkt.

Analytische methoden voor de concentratiebepaling van de topoisomerase I remmers topotecan, 9-aminocamptotecine en lurtotecan in plasma, bloed, urine en feces worden in dit proefschrift beschreven. Daarnaast worden er toepassingen van deze analyse methoden in zowel preklinische als klinische studies beschreven.

In **hoofdstuk 1**, wordt een overzicht gegeven van analyse methoden, zoals die momenteel wereldwijd voor concentratiebepalingen van topotecan, irinotecan, 9-aminocamptothecine en lurtotecan worden gebruikt. Aangezien deze camptothecinederivaten sterk fluorescerende eigenschappen hebben, worden deze stoffen gedetecteerd met behulp van fluorescentie, na te zijn gezuiverd en gescheiden van andere endogeen voorkomende stoffen met behulp van hogedruk vloeistofchromatografie. Hierbij worden componenten op grond van chemische en fysische eigenschappen gescheiden. Aangezien de camptothecinederivaten een zuurgraad afhankelijke omzetting kennen van de lacton- naar de carboxylaatvorm, en visa versa, is het noodzakelijk dat bloedmonsters direct na afname gecentrifugeerd worden om het plasma te verzamelen, waarin de concentraties van de camptothecinederivaten bepaald worden. Daar alleen de werkzame lactonvorm in staat is om celmembranen te passeren, inclusief die van de rode bloedcellen, is het tevens noodzakelijk het bloed direct af te draaien wanneer alleen de totale concentratie van de camptothecinederivaten wordt bepaald. Een verschuiving van het evenwicht tussen de lacton- en carboxylaatvorm in het bloed heeft namelijk een direct gevolg voor de totale concentratie van de camptothecine in het plasma. Nadat het plasma is verzameld kan dit het beste direct ingevroren worden, zodat de omzetting van lacton- naar carboxylaatvorm wordt gestopt. Voor de bepaling van de lactonconcentraties kunnen de plasmamonsters op de dag van analyse ontdaan worden van onder andere eiwitten en de carboxylaatvorm door middel van vaste fase- of vloeistofextracties. In beide gevallen wordt op grond van wateroplosbaarheid

de lacton- van de carboxylaatvorm gescheiden. De totale concentraties van de camptothecinederivaten worden in een tweede analyse gemeten door het aanzuren van het plasmamonster, waardoor de totale hoeveelheid van de camptothecine in de lactonvorm komt. Naast het direct invriezen van plasmamonsters kan het evenwicht tussen de lacton- en carboxylaatvorm ook gestabiliseerd worden door het plasma direct te onteiwitten met koude methanol en dit extract in te vriezen. De lacton- en carboxylaatconcentraties kunnen dan in één analytische bepaling gemeten worden. Doordat de diverse camptothecinederivaten verschillende chemische en fysische eigenschappen hebben, is de laatste methode in de praktijk alleen mogelijk gebleken voor topotecan en irinotecan.

In **hoofdstuk 2** worden de farmaceutische en biomedische aspecten van het wateroplosbare camptothecineanaloog topotecan beschreven. De intraveneuze formulering van topotecan (Hycamtin®) is voor de behandeling van eierstokkanker geregistreerd in Europa en de Verenigde Staten. De orale formulering van topotecan is nog in de fase van klinisch onderzoek.

De gelijktijdige bepaling van de lacton- en carboxylaatvorm van topotecan in humaan plasma, na stabilisatie van het evenwicht door middel van een extractie met ijskoude methanol, en van totale topotecanconcentraties in humane urine is beschreven in **hoofdstuk 2a**. De laagste concentratie die nog adequaat en precies bepaald kon worden, voor zowel de lacton- als de carboxylaatvorm, werd gesteld op 0.10 ng/ml. Hiermee mag deze methode tot één van de gevoeligste worden gerekend. Totale concentraties van topotecan in urine worden gemeten in de lactonvorm na aanzuring van het monster, met een laagste bepalingsgrens van 10 ng/ml.

Een toepassing van de in hoofdstuk 2a beschreven analytische methode is beschreven in **hoofdstuk 2b**. Hierin wordt de invloed van derde ruimtes, zoals ascites en pleuravocht, op de plasmakinetiek van topotecan beschreven bij 4 patiënten. De derde ruimtes fungeerden niet als 'sink' voor topotecan en hadden geen invloed op de farmacokinetiek van zowel de lacton- als carboxylaatvorm van topotecan. De ratio's tussen de concentratie in de derde ruimte en het plasma nam bij alle patiënten toe in de tijd na orale toediening, met een gemiddeld oppervlak onder de concentratie tijdcurve ratio van 0.55. Topotecan kan dus veilig aan patiënten met ascites en/of pleuravocht worden gegeven zonder dat de plasmakinetiek verandert, terwijl er wel farmacologisch actieve concentraties in de ascites en het pleuravocht gevonden werden.

Hoofdstuk 2c beschrijft een klinische fase I studie in 49 patiënten, waarin een vaste dosis cisplatin van 75 mg/m^2 werd gecombineerd met toenemende orale topotecan doseringen van 0.75 tot 2.30 mg/m^2. Cisplatin werd door middel van een 3-uur durend intraveneus infuus toegediend, vlak voor of na een 5-daagse orale toediening van topotecan, welke elke drie weken werd herhaald. Farmacokinetische interacties tussen de twee cytostatica en schema afhankelijke bijwerkingen werden bestudeerd. Cisplatin toegediend voor topotecan gaf aanzienlijk ernstigere bijwerkingen, in de vorm van beenmergschade, dan het omgekeerde schema. De hoogst haalbare

doseringen van topotecan waren respectievelijk 1.25 en 2.00 mg/m^2. In de eerste 18 patiënten werd een mogelijk farmacokinetische interactie van de twee cytostatica onder invloed van het schema onderzocht in een gerandomiseerde 'cross-over' studie. De farmacokinetiek van zowel topotecan als cisplatin werd niet beïnvloed door de volgorde van toediening. In een gerandomiseerde fase II studie zal moeten worden uitgezocht welke van de twee schema's de meeste antitumor-activiteit laat zien.

De meeste cytostatica worden vandaag de dag nog steeds aan de hand van het lichaamsoppervlak van de individuele patiënt gedoseerd. Dit heeft als doel de interpatiëntvariatie in de expositie aan het betreffende cytostaticum te verminderen. In **hoofdstuk 2d** wordt de relevantie van lichaamsoppervlak-gebaseerde dosering van oraal topotecan in volwassen patiënten bediscussieerd aan de hand van de intra- en interpatiëntvariatie in topotecan expositie. De intrapatiëntvariatie in de oppervlakte onder de plasmaconcentratie tijdcurve van topotecan lacton was 24 ± 13% (mediaan 20%, range 7.6 – 61%) in 47 patiënten. De interpatiëntvariatie in de plasmaklaring van de lactonvorm was 38 en 42%, respectievelijk uitgedrukt in L/h/m^2 en in L/h. De kleine variatie in het lichaamsoppervlak van de bestudeerde patiënten van 12% staat in schril contrast tot de gevonden intra- en interpatiëntvariaties in de plasma-expositie en -klaring. Een ander probleem voor exacte toediening van oraal topotecan is het feit dat er alleen capsules beschikbaar zijn met 0.25 en 1.00 mg, zodat alle doseringen sowieso moeten worden afgerond. Oraal toegediend topotecan kan worden toegevoegd aan de lijst met cytostatica, waarbij dosering gebaseerd op lichaamsoppervlak niet beter is dan gelijke dosering van iedere patiënt. Hierbij dient tevens te worden opgemerkt dat een gelijke dosering makkelijker is voor de apotheker en oncoloog, kostenefficiënter is en veel minder omslachtig is voor de patiënt. Om het nut van gelijke dosering bij iedere volwassen patiënt aan te tonen, zal er een gerandomiseerde klinische studie uitgevoerd moeten worden, waarbij de farmacokinetiek, bijwerkingen en tumorrespons worden onderzocht.

Geslachtsafhankelijke verschillen in de plasmakinetiek van topotecan en de verklaring voor dit verschil worden in **hoofdstuk 2e** beschreven. Na orale toediening werd er een significante 1.4 keer snellere plasmaklaring van de lactonvorm van topotecan geconstateerd bij mannelijke patiënten in vergelijking met vrouwelijke patiënten. Na correctie voor de lichaamsoppervlakken bleef de plasmaklaring significant 1.3 keer sneller bij mannen. Daarnaast werd er een significant 1.3 keer hogere ratio bij vrouwen gemeten in de oppervlakte onder de plasmaconcentratie tijdcurven van topotecan lacton tot deze van topotecan totaal. Zowel de hematocriet waarde als het lichaamsoppervlak waren significant verschillend tussen de bestudeerde mannen en vrouwen. Er werd een significante lineaire relatie gelegd tussen de plasmaklaring van topotecan gecorrigeerd voor het lichaamsoppervlak en de hematocriet waarde, terwijl deze relatie niet kon worden gelegd tussen de plasmaklaring en het lichaamsoppervlak. Ook in een studie waarbij topotecan gedurende 21 dagen met een continu infuus werd toegediend, hadden vrouwen een

significant hogere lacton tot totaal 'steady state' concentratieratio. Bij laboratorium-experimenten, waarbij de hematocriet waarde kunstmatig veranderd was, werd een zeer goede significante relatie gevonden tussen de hematocriet waarde en de lacton tot totaal topotecan concentratieratio in het plasmacompartiment van de bloedmonsters. Ook in bloedmonsters van vrouwelijke vrijwilligers werd een significant hogere lacton tot totaal topotecan plasmaconcentratieratio gemeten in vergelijking met bloedmonsters van mannelijke vrijwilligers na incubatie met topotecan. In het plasmacompartiment van de bloedmonsters van vrouwen werd ook een significant hogere concentratie van de lactonvorm gevonden in vergelijking met de lactonconcentratie in de bloedmonsters van mannen. De implicaties van deze geslachtsafhankelijke topotecan plasmakinetiek ten aanzien van de tumorrespons en toxiciteit zullen in een grote studie met geselecteerde patiënten moeten worden bestudeerd, waarbij alle mannen en vrouwen een gelijke dosis topotecan krijgen toegediend.

Hoofdstuk 2f beschrijft een klinische fase I studie met als doel: het ophogen van de dosis topotecan, in vergelijking met de studie die beschreven is in hoofdstuk 2c, in combinatie met een lagere dosering van 50 mg/m^2 cisplatin. De dosislimiterende bijwerkingen waren beenmergsuppressie en klachten betrekking hebbend op het maagdarmstelsel bij een dosis van 1.75 mg/m^2 topotecan gedurende 5 dagen, voorafgegaan door een enkelvoudige dosis cisplatin. Er werden geen verschillen in de plasmakinetiek van zowel topotecan als cisplatin gevonden in vergelijking met eerdere studies. De hoogst haalbare dosis van 1.50 mg/m^2 topotecan gedurende 5 dagen in combinatie met 50 mg/m^2 cisplatin op dag 1 is waarschijnlijk niet effectiever dan het geadviseerde schema van 75 mg/m^2 cisplatin op dag 1 gevolgd door 1.25 mg/m^2 topotecan gedurende 5 dagen. De cisplatin dosering dient namelijk met 33% verminderd te worden om een minimale verhoging in de topotecan dosering mogelijk te maken.

In **hoofdstuk 3** wordt het slecht wateroplosbare camptothecinederivaat 9-aminocamptothecine besproken. Aangezien 9-aminocamptothecine één van de meest effectieve middelen in preklinische modellen bleek te zijn, is er lang gezocht naar een goede formulering voor klinische toepassing. Sindsdien zijn er verscheidene intraveneuze fase I- en II- en orale fase I studies uitgevoerd.

Hoofdstuk 3a beschrijft hogedruk vloeistofchromatografie methoden voor de bepaling van de 9-aminocamptothecine lactonconcentraties en voor de totale concentraties in humaan plasma. De lactonvorm werd geëxtraheerd door middel van een vloeistofextractie, waarbij de laagst adequaat meetbare concentratie 50 pg/ml was. In een tweede analyse werd de totale concentratie bepaald na een simpele onteiwittingsstap, waarbij de totale hoeveelheid 9-aminocamptothecine in de lactonvorm werd gebracht. De laagst adequaat en precies meetbare concentratie bij de laatstgenoemde methode was 100 pg/ml.

Na een 5-minuten durende intraveneuze toediening van 9-aminocamptothecine werd er een zogenaamde 'rebound peak' in het kinetisch profiel van de totale concentratie van 9-aminocamptothecine in humaan plasma geconstateerd. De factoren die deze 'rebound peak' veroorzaken, worden in **hoofdstuk 3b** beschreven. De toegediende lactonvorm van 9-aminocamptothecine bleek in humaan bloed zeer snel omgezet te worden in de niet werkzame carboxylaatvorm, waarbij 3 uur na toediening minder dan 10% zich nog in de lactonvorm bevond. Bij laboratoriumexperimenten, waarbij 9-aminocamptothecine aan vers bloed werd toegevoegd, verdeelde het zich direct over de rode bloedcellen en het plasma. Aangezien de toegevoegde lactonvorm zeer snel werd omgezet in de carboxylaatvorm, die niet in staat is om passief de membranen van de rode bloedcellen te passeren, nam de concentratie van 9-aminocamptothecine in het plasmacompartiment in de tijd toe. In tegenstelling tot onder andere topotecan heeft de carboxylaatvorm van 9-aminocamptothecine een hoge affiniteit voor humaan serumalbumine. Hierdoor komt het evenwicht aan de kant van de carboxylaatvorm te liggen, zodat de totale plasmaconcentraties toenemen. Deze 'rebound peak' werd niet waargenomen bij preklinische studies in muizen. Dit kan verklaard worden doordat de percentages 9-aminocamptothecine die zich - na het instellen van het evenwicht - nog in de lactonvorm bevinden, in oplossingen van humaan- of muizen serumalbumine respectievelijk 0.63 en 35.0% zijn.

In **hoofdstuk 3c** wordt de kinetiek van 9-aminocamptothecine in plasma en speeksel na orale toediening beschreven. Tevens worden relaties gelegd tussen de plasmakinetiek en de bijwerkingen. De lactonvorm van 9-aminocamptothecine wordt, in tegenstelling tot deze andere bekende camptothecinederivaten, zoals topotecan, lurtotecan en irinotecan, zeer snel omgezet in de carboxylaatvorm. De plasmakinetiek was lineair met de dosis met zeer lage intrapatiëntvariaties in de expositie van de lactonvorm, terwijl de interpatiëntvariaties in deze expositie relatief hoog waren. Concentratieratio's tussen ongestimuleerd speeksel en plasma waren zeer variabel, zodat plasma niet vervangen kan worden door ongestimuleerde speeksel voor de bepaling van het kinetisch profiel van 9-aminocamptothecine. Tevens werd bij een patiënt met pleuravocht onderzocht welk effect deze derde ruimte op de plasmakinetiek heeft. Net als voor topotecan (beschreven in hoofdstuk 2b) hoopt 9-aminocamptothecine zich niet in deze derde ruimte op en kan 9-aminocamptothecine aan patiënten met pleuravocht worden gegeven zonder dat de expositie in het plasma afneemt. Ten aanzien van bijwerkingen correleert de oppervlakte onder de plasmaconcentratie tijdcurve van de lactonvorm met het percentage daling in witte bloedcellen en trombocyten. In toekomstige klinische studies met oraal toegediende 9-aminocamptothecine kunnen patiënten individueel gedoseerd worden met een vooraf bepaalde oppervlakte onder de plasmaconcentratie tijdcurve als farmacokinetisch eindpunt, aangezien de intrapatiëntvariatie extreem laag is.

In het één na laatste hoofdstuk van dit proefschrift worden analytische methoden beschreven voor de bepaling van de liposomaal geformuleerde topoisomerase I remmer lurtotecan, terwijl in het laatste hoofdstuk een toepassing van deze methoden wordt beschreven.

Hoofdstuk 4a beschrijft een hogedruk vloeistofchromatografische methode voor de bepaling van totale lurtotecan concentraties in plasma en urine van patiënten na de toediening van NX 211, de liposomaal geformuleerde lurtotecan. Totale plasmaconcentraties worden bepaald na het onteiwitten en aanzuren van het monster, waarbij de laagst adequaat meetbare concentratie op 1.00 ng/ml gesteld is. Deze methode werd ook gevalideerd voor de bepaling van lurtotecan concentraties in urine die hoger zijn dan 100 ng/ml, door de urine voor extractie 40 keer te verdunnen in lurtotecan-vrije plasmamonsters. Voor lurtotecan concentraties in urine lager dan 100 ng/ml werd een andere methode ontwikkeld die gebruik maakt van een vloeistofextractie. Tevens werd het fluorescerende vermogen van lurtotecan bij deze methode verhoogd door het eluaat voor detectie bloot te stellen aan ultraviolet licht, waarbij de piperazinomethyleen groep van het molecuul werd afgesplitst, resulterend in een 14 keer hoger fluorescerend signaal. Concentraties tot en met 0.500 ng/ml konden nog nauwkeurig en accuraat worden gemeten. Deze twee analytische bepalingsmethoden worden gebruikt in een lopende fase I studie met NX 211.

De toepassingen van de hierboven genoemde methoden worden beschreven in **hoofdstuk 4b**, waarin de kinetiek van lurtotecan wordt beschreven bij 6 patiënten die experimenteel NX 211 toegediend hebben gekregen. Tevens worden twee nieuwe methodieken beschreven voor de bepaling van lurtotecan concentraties in bloed- en fecesmonsters. De monstervoorbewerking bestaat voor beide methoden uit een onteiwittingsstap in combinatie met aanzuren. Doordat ook hier het fluorescerend vermogen werd verhoogd door middel van ultraviolet licht, werden de laagst adequaat meetbare concentraties in bloedmonsters en gehomogeniseerde fecesmonsters respectievelijk gesteld op 0.25 en 10 ng/ml. De plasmakinetiek van lurtotecan kon in alle gevallen het best worden beschreven met een mono-exponentiële afname, met een halfwaarde tijd van gemiddeld 6 uur. De plasmaklaring was gemiddeld 0.78 L/uur/m^2, wat 25 keer langzamer is dan de plasmaklaring van niet liposomaal toegediende lurtotecan. Het distributie volume op "steady state" was ongeveer net zo groot als het totale bloedvolume van de patiënten en de bloed tot plasma totaal lurtotecan concentratieratio was gemiddeld 0.66. Deze parameters zijn indicatief dat lurtotecan zich nog in de liposomen bevindt, die zich voornamelijk in het plasmacompartiment ophouden. Zowel de urinaire als de fecale excretie van onveranderd lurtotecan was laag met respectievelijk gemiddelde waarden van 8.6% en 9.7%. Een groot deel van lurtotecan zal dus waarschijnlijk omgezet worden in metabolieten. In de chromatogrammen van gehomogeniseerde fecesmonsters werden 2 extra pieken gevonden, die afwezig waren in de blanco fecesmonsters van de patiënten. Momenteel wordt in een lopende fase I studie gekeken naar de toxiciteit en mogelijke anti-tumor respons in relatie tot de farmacokinetiek.

Conclusies en toekomstdromen

De camptothecinederivaten behoren tot de klasse van de topoisomerase I remmers, die niet meer weg te denken zijn uit de kankergeneeskunde. Effectiviteit is onder andere aangetoond tegen eierstok- en dikkedarmkanker. Om de concentraties van deze topoisomerase I remmers in verschillende biologische monsters adequaat te kunnen bepalen zijn vele methodieken ontwikkeld die voornamelijk gebaseerd zijn op hogedruk vloeistofchromatografie in combinatie met fluorescentie detectie. Aangezien alleen de lactonvorm werkzaam is, en het evenwicht tussen de lacton- en carboxylaatvorm in het plasmacompartiment varieert tussen de verschillende analoga en kan variëren van mens tot proefdier, blijven goede analysemethoden voor de bepaling van de lactonvorm zeer belangrijk om goede relaties te kunnen leggen tussen de (plasma)kinetiek van een camptothecine en de bijwerkingen en/of tumorrespons.

Ter verhoging van de effectiviteit zullen in de toekomst de topoisomerase I remmers steeds vaker worden gecombineerd met cytostatica met andere werkingsmechanismen. Aangezien in combinatietherapieën de doseringen van de cytostatica meestal lager zijn, blijven gevoelige, selectieve, accurate en reproduceerbare bepalingsmethoden nodig om inzicht te krijgen in mogelijke farmacokinetische interacties en de hieruit voortvloeiende bijwerkingen en/of tumorrespons.

Ik droom van een toekomst waarin anti-kankermiddelen individueel worden gedoseerd, gebaseerd op het farmacokinetisch profiel van het betreffende cytostatica in iedere afzonderlijke patiënt, met het doel om de bijwerkingen te verlagen, terwijl de effectiviteit van het middel verbetert. Met het oog op de vorderende kennis van genetische verschillen in onder andere enzymsystemen, verantwoordelijk voor bijvoorbeeld detoxificatie van allerhande stoffen, zou de realiteit van deze droom toch niet heel ver weg hoeven te zijn

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 12 november 1968 te Rotterdam. Zijn jeugd heeft hij doorgebracht in Nieuw-Lekkerland, alwaar hij de lagere school doorliep. In 1986 behaalde hij het HAVO eindexamen aan de Lage Waard te Papendrecht. In Rotterdam werd in hetzelfde jaar begonnen aan de opleiding tot medisch microbiologisch analist aan de polytechnische faculteit van de Hoge School Rotterdam & Omstreken, welke in 1990 met goed gevolg werd beëindigd. In het kader hiervan was hij gedurende het laatste studiejaar werkzaam op de afdeling virologie van het Academisch Ziekenhuis Rotterdam (Prof. dr. N. Masurel). Onder leiding van de viroloog dr. Ph.H. Rothbarth en hoofdanalist P. Schrijnemakers is de liefde voor de research ontstaan. Na het vervullen van de militaire dienst is hij in november 1991 als research analist in dienst getreden van de afdeling interne oncologie (Prof. dr. G. Stoter) van Dr. Daniel den Hoed Kliniek op het laboratorium van de experimentele chemotherapie en farmacologie (Prof. dr. J.H.M. Schellens), alwaar hij in de gelegenheid is geweest zelfstandig onderzoek te verrichten. De laatste jaren was dit onderzoek, onder leiding van Prof. dr. J. Verweij en dr. A. Sparreboom, voornamelijk gericht op topoisomerase I remmers, wat uiteindelijk geresulteerd heeft in dit proefschrift.

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