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Clinical study

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

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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 h 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. [© 1999 Lippincott Williams & Wilkins.]

Key words: 9-Amino-20(S)-camptothecin, clinical pharmacokinetics, erythrocytes, *in vitro*, proteins, rebound concentrations.

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 analogs, including 9-AC, were previously shown to be complicated by a chemical, pH-dependent instability of the terminal α-hydroxy-δ-lactone ring (Figure 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 h.[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

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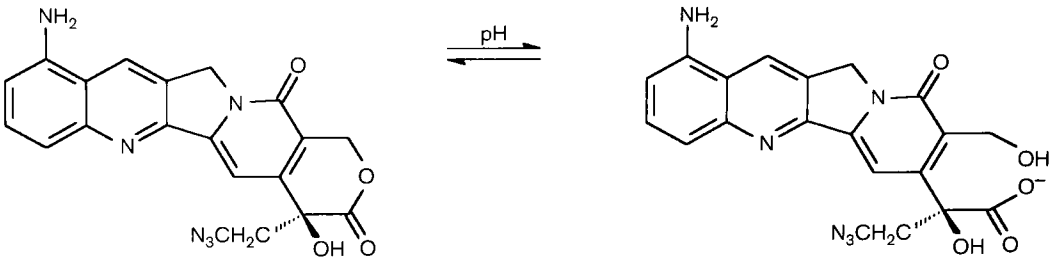

Figure 1. Chemical structures and pH-dependent interconversion of the lactone and carboxylate forms of 9-AC.

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.

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 (St Louis, MO). 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).

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 was 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 PBS, and in heparinized human whole blood and plasma. 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; Gibco/BRL, Life Technologies, Breda, The Netherlands), supplemented with 10% of heat-inactivated fetal calf serum (Hyclone, Logan, UT), 10 mM of sodium carbonate, 2 mM 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 80–90% confluence in six-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 3 times with ice-cold PBS 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 was 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 h after drug administration (Figure 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 0.1–1.0 μg/ml (Figure 3). 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 h (Figure 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 (about 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,

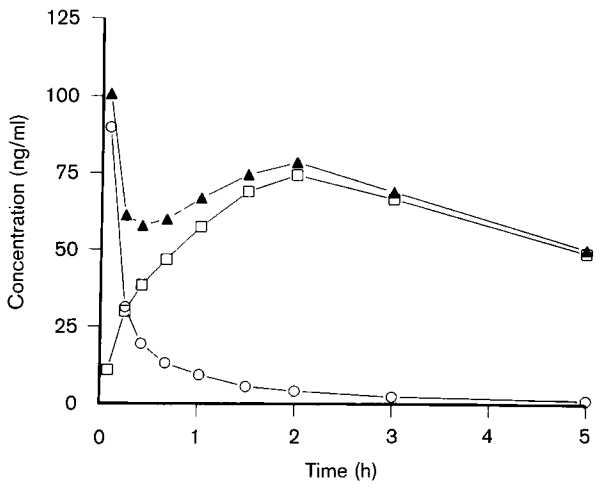

Figure 2. Representative plasma concentration–time profile of 9-AC lactone (○), carboxylate (□) and total drug (i.e. lactone plus carboxylate forms;▲) in a single patient after i.v. drug administration of 1.0 mg/m^2.

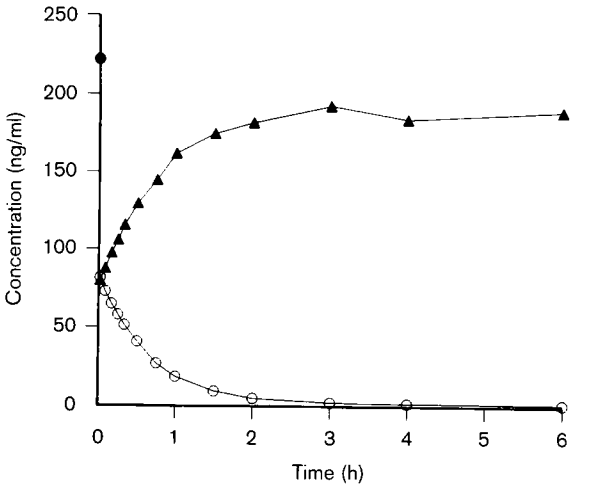

Figure 3. Extracellular concentration–time profiles of 9-AC lactone (○) and 9-AC total drug (▲) following incubation of 9-AC lactone at 100 ng/ml in human whole blood. The closed circle on the ordinate indicates the initial 9-AC total extracellular concentration estimate, based on a hematocrit of 0.45.

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)	Percent as lactone at equilibrium	pH
PBS	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]Half-life.

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

Cell line	9-AC lactone (ng/mg protein)	9-AC carboxylate (ng/mg 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 h 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 PBS (to a hematocrit of about 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. 30 min or less). 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 the 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. below 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 the 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 $1.30 \pm 0.50\%$, 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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