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Pharmacokinetics of the *in Vivo* and *in Vitro* Conversion of 9-Nitro-20(*S*)-camptothecin to 9-Amino-20(*S*)-camptothecin in Humans, Dogs, and Mice[1]

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Abstract

We have determined that 9-nitro-20(*S*)-camptothecin (9NC) converts to 9-amino-20(*S*)-camptothecin (9AC) in humans, dogs, and mice. Following a single oral dose of 0.1 mg/kg of 9NC, the human plasma concentration reached a maximum concentration of 483 ng/ml at 3.4 h with an area under the curve (AUC) of 2.6 μg·h/ml and a half-life of 2.5 h. As conversion of 9NC to 9AC occurred, the maximum calculated concentration of 9AC was 14.0 ng/ml at 10.3 h with an AUC of 311 ng·h/ml and a half-life of 7.1 h. Following a single oral dose of 1.0 mg/kg of 9NC, the maximum concentration of 9NC in the human volunteer was 1247 ng/ml at 5.3 h with an AUC of 17194 ng·h/ml and a half-life of 4.9 h. In this human, the C_{max} of 9AC was 208 ng/ml at 17.2 h; the AUC was determined to be 9121 ng·h/ml, and the half-life was 13.1 h.

In a dog after a single oral dose of 1.0 mg/kg 9NC, the maximum concentration for 9NC was 19.1 ng/ml at 0.7 h with a half-life of 6.4 h and an AUC of 186 ng·h/ml. The maximum concentration of 9AC in this dog was 9.2 ng/ml at 2.9 h with an AUC of 310 ng·h/ml and a half-life of 21.1 h.

The maximum concentration of 9NC in the mouse after a single oral dose of 4.1 mg/kg of 9NC was 732 ng/ml at time 0.1 h with an AUC of 441 ng·h/ml and a half-life of 10.0 h. The maximum concentration of 9AC in the mouse was 26 ng/ml at 0.6 h. The AUC was 63 ng·h/ml, and the half-life was 1.2 h.

Incubation of mouse liver, spleen, kidney, brain, and muscle tissue with 9NC all indicated conversion to 9AC, yet no conversion was observable in cell-free plasma from human or mouse blood. Structural identification of 9AC was confirmed by mass spectrometry.

Introduction

CPT[3] (1) has been a focus of attention in cancer research for almost three decades, since it first was shown to have considerable antitumor activity against L1210 mouse leukemia (1). Unfortunately, the CPT derivative selected for clinical trials was the sodium carboxylate salt, used because it was water soluble and could be injected via the i.v. route. In this form, the CPT molecule produced considerable toxicity and seemed devoid of anticancer activity (2–6) as we have also shown in our laboratory (7). Several CPT analogues have been synthesized over the years (8–17), but interest in camptothecin and its derivatives suddenly increased after it was discovered that camptothecin inhibits topoisomerase I (18–21). Using native CPT with the closed lactone ring intact, we have demonstrated in our laboratory that the oral and intramuscular routes of drug administration are superior to i.v. injections. Thus, we were able to maximize the antitumor activity and eliminate much of the severe toxicity which was observed with the water-soluble carboxylate form (22). At the same time, we reported (7) that 9AC and 9NC showed greater antitumor activity than CPT. When establishing the plasma levels of 9NC after oral administration to dogs, we found that 9NC converts to 9AC. In this report, we describe the results of our studies on this conversion in different species *in vitro* as well as *in vivo*.

Materials and Methods

Chemicals

CPT was imported from Good Land Enterprises, Ltd. (Vancouver, British Columbia, Canada) or purchased from Sigma Biochemicals (St. Louis, MO). Regardless of the source, CPT was purified to 99% for all studies. Acetic acid, acetonitrile, chloroform, methanol, ammonium acetate, sodium azide, *N,N*-dimethylacetamide were ACS quality reagents and obtained from Aldrich Chemical Co. (Milwaukee, WI). Thin layer chromatography plates were from Eastman Kodak Company (Rochester, NY). Absolute ethanol (200 proof) was from AAPER Alcohol Co. (Shelbyville, KY). Perchloric acid was from Fisher Scientific (Pittsburgh, PA). HPLC grade water was prepared in-house using the Mille-Q μf Plus system from Millipore (Bedford, MA). HPLC buffers were filtered through a 0.45-μm nylon filter from Schleicher and Schuell (Keene, NH) before use. Reduced pentacarbonyl iron was from Sigma Biochemicals. RPMI 1640 tissue culture medium was without fetal calf serum.

Instrumentation

The HPLC system consisted of a Beckman 421 controller with two 110A pumps and a 2000-μl injection loop. The UV and the fluorescence detectors were SPD-110AV and RF 551, respectively. Both detectors were from Shimadzu (Kyoto, Japan). Reverse phase HPLC analysis of the samples was carried out by using a mobile phase consisting of 23% methanol:acetonitrile (3:7) and 77% of 10 mM of ammonium formate at pH 1.95. Analyses were carried out at room temperature with a flow rate of 1 ml/min. The HPLC detectors were connected in series and set to monitor the UV absorbance at 220 nm. The fluorescence detector excitation was set to 360 nm, and the emission was monitored at 454 nm. The integrating software used was EZChrome (Shimadzu) and FLO-ONE\Beta (Radiomatic Instruments, Meridian, CT). A dual-channel model BD112 flatbed recorder was from Kipp and Zonen (Bohemia, NY). The samples were usually analyzed on a C_8-Microsorb MV HPLC column or on a cyano or a C_{18}-Microsorb MV HPLC column (Rainin Instruments, Woburn, MA). Samples were collected for further analysis with a HeliFrac from Pharmacia (Piscataway, NJ). Samples were weighed on a model H10T by Mettler Instrumentation Corporation (Highstown, NY). Pharmacokinetic computations were carried out with RSTRIP II software from Micromath Scientific Software (Salt Lake City, UT). The normal and the tandem mass spectra were taken on a ZAB-SEQ Mass Spectrophotometer from V. G. Analytical of Manchester, England at Baylor College of Medicine, Department of Experimental Therapeutics. Dogs were obtained from St. Joseph Surgical Training Center (Houston, TX). Swiss nude mice were bred in our laboratory as described before (23).

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[3] The abbreviations used are: CPT, camptothecin; 9AC, 9-aminocamptothecin; 9NC, 9-nitrocamptothecin; HPLC, high performance liquid chromatography.

Drug Administration

Drug administration was carried out orally in dogs and humans by packaging the drug in a gelatin capsule. In the mouse experiment, CPT was given by the oral route by suspending the drug in cottonseed oil at a concentration of 4.0 mg/kg/0.1 ml, followed by administration of 0.1 ml by trochar. Nude mice of Swiss background weighed approximately 30 g. The dog was healthy and weighed 20 kg. After obtaining permission from the St. Joseph Hospital Medical Research Committee (Institutional Review Board), a healthy human volunteer (B. C. G.), weighing 90 kg, received a single oral dose of 9NC at 0.1 mg/kg and 1.0 mg/kg with a three-week interval between each dose.

Chemistry

HPLC Conditions. Using a C_8 column, the mobile phase consisted of 23% acetonitrile:methanol (7:3) and 77% of 10 mM ammonium formate (pH 1.95) for all determinations. The detection limit under these conditions was 0.25 ng/ml when analyzing a sample for 9AC only. When the amount of total drug was measured on HPLC following the conversion of 9NC to 9AC in plasma, the detection limit dropped to 0.5 ng/ml, probably owing to the needed extra manipulation.

***In Vitro* Conversion of 9NC to 9AC in Dog Liver.** Fresh liver from an adult dog (555.4 g) was weighed and dissected. Of this mass, 278.4 g was removed and combined with 600 ml of RPMI media without fetal calf serum and homogenated in a Singer Turboblend 7 blender for approximately three min. A 50-ml fraction was separated and used for the control experiment. The remaining mixture was transferred into a container and stirred magnetically. A solution of 25 mg of 9NC in 15 ml of an aqueous ethanol solution (33%) containing 0.1% acetic acid was added. The mixture was incubated at 37°C. Fractions were collected at 0, 0.25, 0.5, 1, 2, 3, 4, and 16 h. Samples were analyzed for total drug only without attempting to separate lactone and carboxylate forms of either 9NC or 9AC. Therefore, each sample was prepared for analysis by HPLC by taking a 100-μl fraction of the homogenate. To this was added 10 μl of 1.5 M perchloric acid, followed by vortexing the mixture for 20 s, followed by equilibration at room temperature for 10 min. Next, 300 μl of methanol were added, and the sample was vortexed for 10 s, centrifuged at 13,000 × *g* for 2 min, followed by removal of the supernatant. The supernatant was added to 1000 μl of 10 mM ammonium formate with a pH 1.95. A volume of 50 μl was injected into HPLC. The retention time for 9AC was 6.1 min. The same technique was followed for the determinations in mouse organs (Table 1).

***In Vivo* Determination of 9AC Plasma Levels.** Perchloric acid (10 μl) was added to 100 μl of plasma sample. The mixture was vortexed for 10 s and left at room temperature for 10 min. Methanol (300 μl) was added, and the mixture was vortexed for 10 s, followed by centrifugation at 13,000 × g for 2 min. The supernatant was removed and added to 1000 μl of 10 mM ammonium formate (pH 1.95). Of this solution, 500 μl was injected through a 2-ml injection loop onto a Rainin Microsorb C_8 column. The 9AC concentration was determined by measuring the fluorescence area using an excitation wavelength of 360 nm and an emission wavelength of 454 nm and comparing the spectral peaks to the previously established standards. These standards were calculated according to the formula:

$$\text{ng/ml} = \frac{\text{Fluorescence area} - A}{B}$$

with the constants of $A = 4538.14$ and $B = 835.04$. The correlation coefficient was 0.9977, and the value for R^2 was 0.9955. Plasma samples were obtained at different time points for humans, dogs, and mice. Since the overall fluorescence of 9NC is less than the fluorescence of 9AC, the *in vivo* content of 9NC in the blood plasma was determined by reducing 9NC *in situ*. This allowed for the determination of the total drug concentration (9NC plus 9AC). The levels of 9NC were then determined by subtracting the previously determined 9AC levels.

Procedure for the Determination of Total Drug. To 100 μl of plasma sample was added 20 μl of an aqueous iron solution consisting of 5 ml of water and 100 mg of reduced pentacarbonyl iron, followed by the addition of 20 μl of concentrated HCl. The mixture was vortexed for 10 s and allowed to remain at room temperature for 10 min. Then 300 μl of 10% ammonium hydroxide in methanol was added, followed by vortexing for 10 s and equilibrating for 5 min. The plasma was centrifuged for 2 min at 13,000 × *g*. The supernatant was drawn off and added to 1000 μl of 10 mM ammonium formate (pH 1.95). Perchloric acid (100 μl; 1.5 M) was then added and vortexed. Of this solution, 500 μl was injected into a 2-ml injection loop onto a Microsorb C_8 column. The total drug concentration was again measured by comparing the peak areas to the previously established 9NC standards.

Table 1 *Distributions of 9NC and 9AC in tissue*

The relative conversion of 9NC to 9AC in five different tissues. Tissues were minced and further homogenized in a 2-ml Pyrex tissue grinder. To 1 g of homogenized tissue was added 5 ml of RPMI solution without fetal calf serum containing 2500 ng of 9NC. This solution was incubated at 37°C. Aliquots were taken out after period of time, extracted by the established procedure, and injected onto HPLC.

Tissue	Time (h)[a]	% conversion[b]	9AC (ng/ml)[c]
Brain	24	63.4	317
Liver-fresh	1	59.8	299
Liver-boiled	0.15	0	0
Spleen	30	57	285
Muscle	24	67.4	337
Kidney	1	75.6	37.8
Whole blood	24	Trace	Trace
Plasma	24	0	0

[a] Time after which the maximum concentrations were observed.
[b] Value reached for the maximum percentage of conversion of 9NC to 9AC.
[c] Measured amounts 9AC in ng/ml. No conversion was observed in blood plasma.

It should be noted that the position and the plasma concentrations of 9NC and 9AC were also confirmed by the above experiment using different mobile phases. In another experiment, the peak corresponding to 9AC was isolated using the C_8 column and reinjected onto a C_{18} column. Injection of a previously collected 9AC peak onto a cyano column also resulted in retention times and the fluorescence intensities that corresponded to the 9AC standard. Furthermore, a plasma peak corresponding to 9AC was isolated using the C_8 column and reinjected under gradient conditions, whereby 10% of the above aqueous buffer was ramped up to 50% over a period of 40 min. In all cases, the identity and quantity of 9NC and 9AC was confirmed (data not shown).

Pharmacokinetic Analysis

The pharmacokinetic parameters were based on the intensities in the fluorescence spectrum. The plot of the plasma concentration *versus* time was used to calculate the terminal rate constant (k) by means of a logarithmic-linear analysis. All integrals were calculated to infinity. The least squares minimization techniques were carried out using the Powell algorithm. The half-life values were calculated using the rate constant (k) in the equation $t_{1/2} = 0.693/k$. The calculated value for the area under the curve was accepted when an independent calculation of the trapezoidal integral agreed with the value obtained for the area under the curve, allowing a deviation of less than 5%. The best curve fitting was obtained for all three vehicles when second order kinetic models were used.

Mass Spectrometric Analysis

The biological fractions from the dog liver incubation experiment that corresponded to the peak of 9AC were pooled from HPLC and lyophilized. Storage was carried out under nitrogen at −70°C until analysis, at which time the samples were reconstituted in methanol. Fig. 1 shows the fragmentation pattern of the tandem mass spectrum of the biological liver sample with a m/e 363.[4] $[C_{20}H_{17}N_3O_4]^{\cdot+}$. The top graph of Fig. 2 shows the fragmentation pattern of the biological sample, and below it is shown an authentic sample of 9AC as prepared in our laboratory, showing that both samples are indeed identical.

Results

Pharmacological Results. In general, our efficiency of converting 9NC to 9AC in either organic or aqueous solution was about 100%.

[4] J. S. Stehlin, E. A. Natelson, H. R. Hinz, B. C. Giovanella, P. D. De Ipolyi, K. M. Fehir, T. P. Trezona, D. M. Vardeman, N. J. Harris, A. K. Marcee, A. J. Kozielski, and A. Ruiz-Razura. Phase I clinical trial and pharmacokinetics results with oral administration of 20(*S*)-camptothecin, submitted for publication.

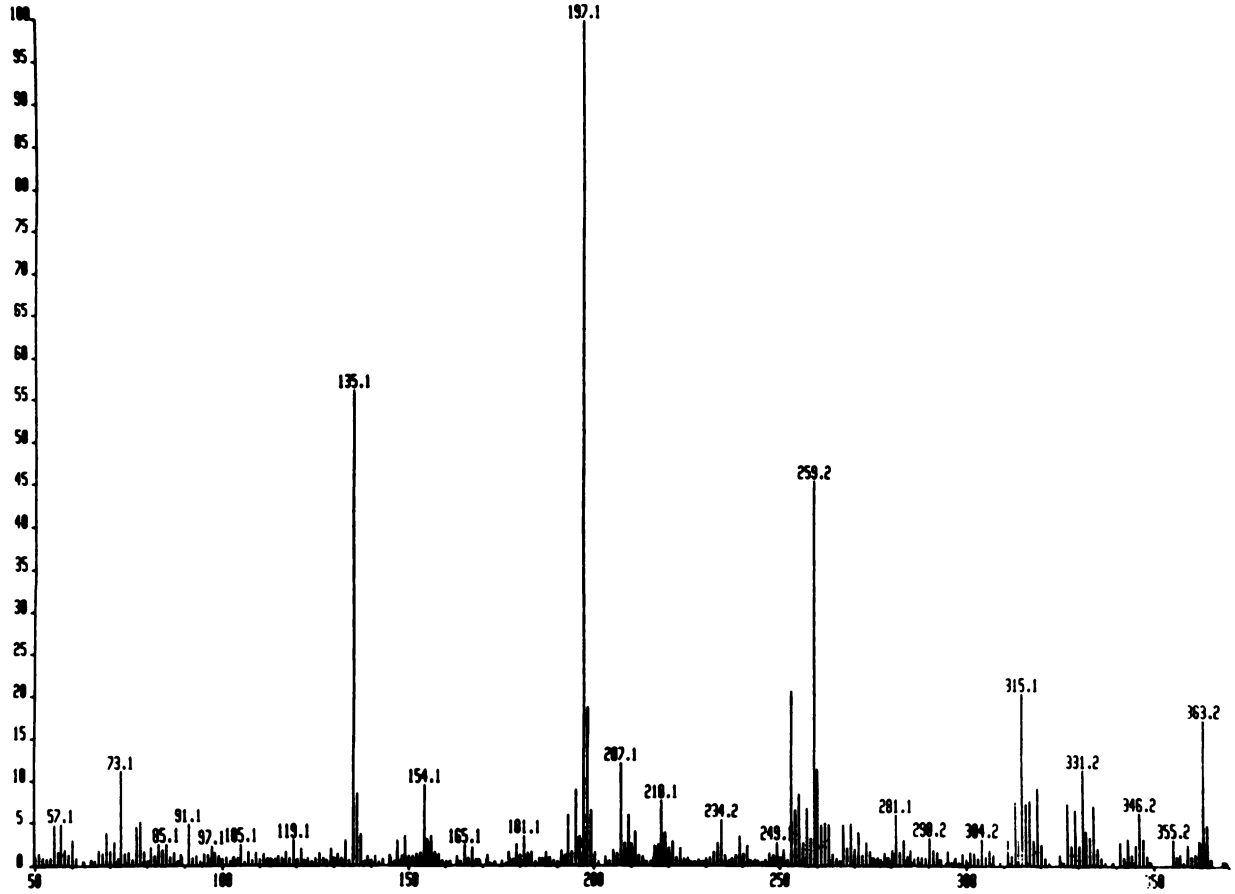

Fig. 1. Shows the detailed mass spectrum of the biological blood plasma sample. The molecular ion appears at 363.2, corresponding to the molecular formula of 9AC.

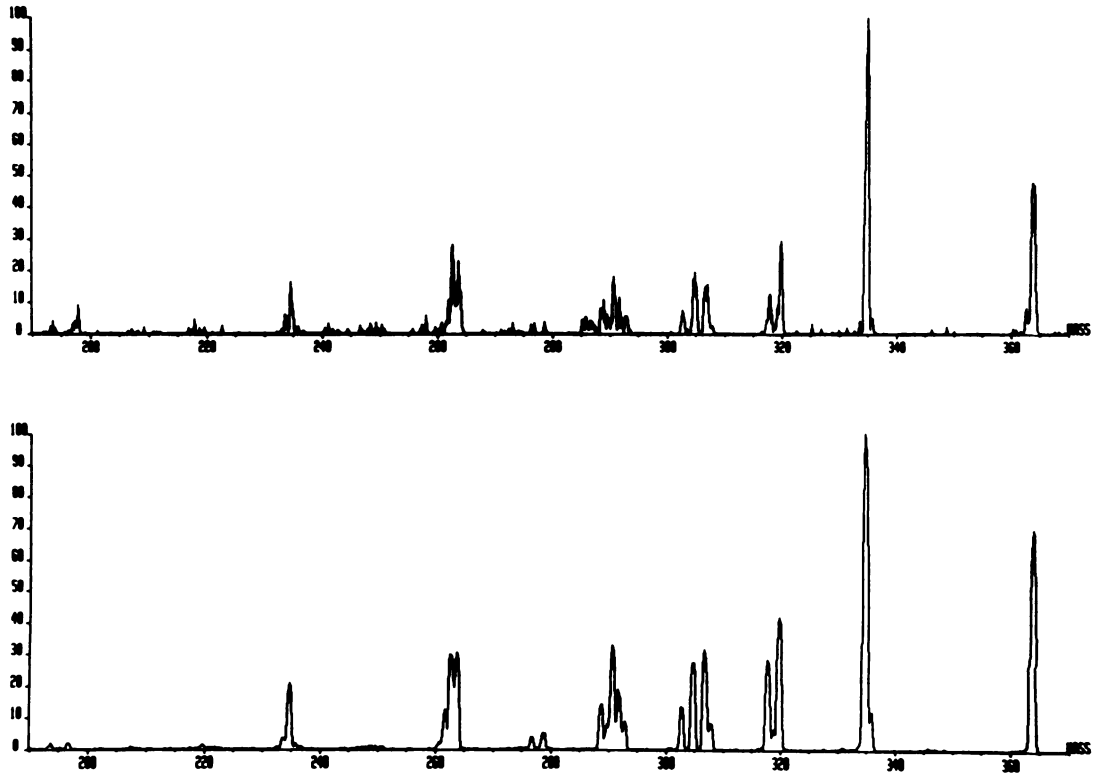

Fig. 2. *Top*, fragmentation pattern of the plasma sample. *Bottom*, an authentic 9AC sample was synthesized in our laboratory by reduction of 9NC to 9AC. The tracings reveal the same identity of both samples.

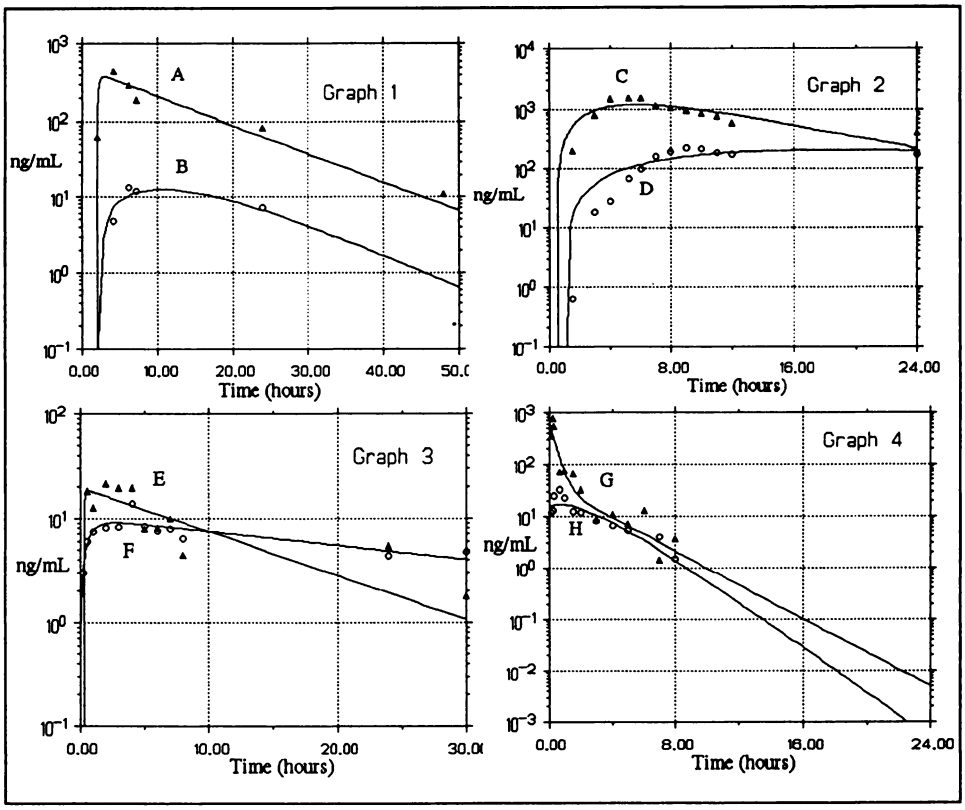

Fig. 3. Elimination curves after oral administration of 9NC. All four tracings use the Y-log scale to show the amounts of 9NC and 9AC in the blood plasma. The *Graph 1* (*upper left*) shows human plasma after a single dose of 0.1 mg/kg. *Curve A* shows levels of 9NC, and *Curve B* shows 9AC concentration. The curve was calculated with a weight factor of 0.8. *Graph 2* (*upper right*) shows human plasma concentration after single dose of 1.0 mg/kg. *Curve C* shows the levels of 9NC, and *Curve D* shows 9AC concentration. The curve was calculated with a weight factor of 0.2. In *Graph 3* (*lower left*), the dog plasma concentration is shown after single dose of 1.0 mg/kg. *Curve E* shows the levels of 9NC, and *Curve F* shows 9AC concentration. The curve was calculated with a weight factor of 0.2. In *Graph 4* (*lower right*), the mouse plasma concentration is shown after administration of a single dose consisting of 4.0 mg/kg. *Curve G* shows the levels of 9NC, and *Curve H* shows 9AC concentration. The curve was calculated with a weight factor of 1.0.

Our experimental efficiency of monitoring the 9NC conversion by first reducing the plasma sample *in situ*, followed by the extraction, was approximately 90%. The results of the pharmacokinetic analysis of the blood plasma samples are summarized in Table 2 and graphically displayed in Fig. 3. When the three systems of humans, dogs, and mice are compared, it becomes obvious that their metabolism of 9AC and 9NC are quite different. This is most obvious when comparing the elimination curves of 9AC and 9NC to each other, wherein it was found that the mouse has the ability to eliminate 9NC and 9AC the quickest. When comparing the curves and the calculated pharmacokinetic values of the human high dose (1.0 mg/kg) with its low dose (0.1 mg/kg), it becomes evident that: (*a*) the blood does get saturated with 9NC only at higher dose levels; and (*b*) the drug stays longer in the blood. This can be seen from Fig. 3, *curves C* and *D*, where both curves meet at approximately 24 h. We suspect that this slow elimination after saturation with 9NC and 9AC at 1.0 mg/kg was responsible for the observed toxicity in the human model. The same crossing of the curves was seen in dogs (Fig. 3, *curves E* and *F*), but here no toxicity was observed.

Table 2 *Pharmacokinetic results for human, dog, and mouse*

Pharmacokinetic results for human, dog, and mouse. The best curves for the model data points were obtained when the data were fit for a biexponential curve.

In vivo system[a]	C_{max} (time in hours)[b]	AUC[c]	$t_{1/2}$ (hours)
Human-9NC (0.1)	483 (3.4)	2,599	2.5
Human-9AC (0.1)	14.0 (10.3)	311	7.1
Human-9NC (1.0)	1,247 (5.3)	17,194	4.9
Human-9AC (1.0)	208 (17.2)	9,121	13.1
Dog-9NC (1.0)	19.1 (0.7)	186	6.4
Dog-9AC (1.0)	9.2 (2.9)	310	21.1
Mouse-9NC (4.1)	732 (0.1)	441	10.0
Mouse-9AC (4.1)	26 (0.6)	63	1.2

[a] Type of test system used. The numerical values in parentheses give the magnitude of the single dose in mg/kg.
[b] Area under the curve in ng·h/ml.
[c] Maximum plasma concentration in ng/ml.

Toxicity. The human toxicity observed at the higher dose of 1.0 mg/kg was total alopecia with a drop in the total leukocyte count from 5,300/mm^3 to 3,000/mm^3, and a fall in the platelet count from 230,000/mm^3 to 70,000/mm^3. Hematological recovery occurred within a week. The lower dose of 0.1 mg/kg of 9NC did not affect the blood count. No animal toxicity was observed. This problem of toxicity should be clinically manageable, however, since we have observed good antitumor activity at much lower levels[5] in animal models.

A major problem with 9AC is that its chemical synthesis, using the semisynthetic method, is carried out by nitration of CPT, followed by reduction to the amino group. This is a low yield operation. In addition, 9AC is light sensitive, heat sensitive, and oxygen sensitive. This makes the production and stabilization of 9AC difficult. The decomposition reactions of 9AC lead to compounds that exhibit a large degree of toxicity in nude mice, whereas the toxicity of pure 9AC is significantly less.[5] We were, therefore, interested in the use of 9NC as a clinical alternative to 9AC, because of its easier availability and greater stability.

Our results from the incubation experiment are summarized in Table 1. From this experiment, we suspected that this reaction could be enzymatic in nature and probably due to the action of a reductase. This conclusion is supported by the observation that boiling the above liver homogenates for 10 min eliminated the ability to cause the conversion of 9NC. Further, incubation of 9NC with fresh human plasma yielded no conversion to 9AC, even after a 48 h incubation period. All of the other tested tissues (Table 1) showed appreciable conversion of 9NC to 9AC, complicating the overall pharmacokinetic picture.

[5] Unpublished results.

Discussion

We believe that 9NC would make an excellent choice for use in a Phase I clinical trial for several reasons: (*a*) its antitumor activity is greater by a factor of 3 than the antitumor activity of CPT in our nude mouse model; (*b*) the pharmacological effects in the human of a single dose (1.0 mg/kg) show a prolonged therapeutic interval available with this drug. 9NC has shown anticancer activity at plasma concentrations as low as 1 ng/ml maintained for 24 to 96 h, using our human xenograft model. We have observed that we can maintain 100 times this concentration for 48 h in a human subject with a peak concentration equaling over 1000 times the therapeutic dose and the observation of only minimal toxicity. For instance, we did not see any signs of cystitis or diarrhea as we saw during our clinical study with CPT. We would expect to use lower amounts of 9NC in order to obtain good antitumor effects. We are now investigating whether the anticancer activity seen after 9NC administration is due to 9NC itself or by the 9AC generated by the conversion reaction described above.

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