Pharmacological Determinants of 9-Aminocamptothecin Cytotoxicity

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ABSTRACT

The camptothecins are a group of anticancer agents with a unique mechanism of action: poisoning of eukaryotic DNA topoisomerase I. 9-aminocamptothecin (9-AC), a potent water-insoluble derivative of camptothecin, is currently undergoing clinical testing. The kinetics of the active derivative 9-AC lactone in cell culture media was defined, and then 9-AC cytotoxicity against human breast (MCF-7), bladder (MGH-U1), and colon (HT-29) cancer cell lines was studied. The relationship between cytotoxic effects, drug concentration, and exposure time was then explored. For all of the three cell lines, 9-AC cytotoxicity increased with both higher drug concentrations and longer exposure times. However, when the duration of exposure was less than 24 h, cytotoxicity was limited and less than 1 log of cell killing occurred, even with very high drug concentrations. Minimal cell killing was also observed unless 9-AC concentrations exceeded a threshold of 2.7 μM. No fixed relationship between the survival fraction and the area under the drug concentration-time curve could be modeled that would fit all of the three cell lines. However, data for the three cell lines from the multiple exposure time experiments were fitted very well to the pharmacodynamic model \( C^n t = k \) \((n^2, 0.90–0.99)\), where \( C \) is the drug concentration, \( n \) is the drug concentration coefficient, and \( t \) is the exposure time. For the three cell lines, to kill 1 log of cells, \( 0.30 < n < 0.85 \), which indicated that duration of exposure was more important than concentration. Our data support the use of 9-AC by infusion for 24 h or longer in clinical studies providing target plasma concentrations can be achieved.

INTRODUCTION

9-AC\(^2\) is a water-insoluble derivative of CPT, an anticancer agent derived from the stemwood of the Chinese tree *Camptotheca acuminata* by Monroe Wall and colleagues in 1966 (1). CPT was found to inhibit the growth of a wide range of experimental tumors *in vitro* (2–5). Because CPT was water-insoluble, the water-soluble derivative sodium CPT was used in early clinical trials. Clinical evaluation was discontinued in the early 1970s because of unpredictable serious toxicity and low antitumor activity (6, 7). Subsequent studies found that an intact lactone ring (E ring) with an “S” stereoisomeric configuration at carbon 20 is essential for antitumor activity (4, 8, 9). The CPT lactone undergoes hydrolysis to produce a water-soluble hydroxy acid or carboxylate form, which is much less potent as an inhibitor of topoisomerase I and as an anticancer agent (4, 8–10). The lactone and carboxylate forms of CPTs exist in a pH-dependent equilibrium. Interest in the CPTs was renewed in the late 1980s after reports that the enzyme topoisomerase I was its major cellular target (11). A series of CPT derivatives with increased potency and lower toxicities compared with the parent compound have been synthesized and evaluated (4, 12, 13). Derivatives of CPT currently in clinical study include 9-AC, topotecan, 9-nitrocamptothecin, and irinotecan (CPT-11).

There has been considerable research effort into the interdependent relationship of both drug concentration and time on antitumor activity. Of particular interest is whether clinical response can be correlated with a function of concentration and time such as the plasma AUC (14). Several studies have found that the correlation between cytotoxicity and \( C \times t \) is often imperfect (15, 16). One reason for failure to observe a consistent dependence of antitumor activity on \( C \times t \) is explained by the equation \( C^n t = k \) (17). In this equation, \( C \) is the drug concentration, \( t \) is the exposure time, \( n \) is the drug concentration coefficient, and \( k \) is the drug exposure constant. When the exponent \( n \) is \(<1\), this indicates a greater importance for exposure time, whereas \( n \geq 1 \) indicates a greater importance for concentration (18, 19). This relationship was first proposed to describe the effect of disinfectants on bacteria. The relevance of this relationship to antitumor agents was realized by Skipper (17), who used this relationship to describe the effect of antitumor agents on reducing a leukemic cell population. Recently, Adam et al. (18) proposed the use of this equation to calculate the \( C_{min} \) and \( t_{min} \) required to achieve a therapeutic end point. These parameters represent the minimum conditions required to produce a certain level of antitumor effect and are useful when comparing the effect of different antitumor agents or a single agent on different patients considering both the extracellular concentration and exposure time rather than concentration alone.

Although a number of studies have examined the cytotoxicity of 9-AC against human cancer cells (20, 21), few studies have studied the relationship between cytotoxicity and drug concentrations, especially the relationship between the cytotoxicity, concentration, and exposure time. Researchers have found that exposure time is important for the cytotoxicity of 9-AC; however, the exact model of the Effect-Concentration-Time relationship has not yet been established. The main purpose of.
this project was to investigate the PK/PD relationship of 9-AC against human cancer cells. Once this relationship is established, it would provide a useful PD framework for additional in vitro and in vivo studies.

MATERIALS AND METHODS

Cells. Experiments were performed with the human cancer cell lines MCF-7 (breast), MGH-U1, (bladder), and HT-29 (colon). All of the lines were obtained originally from the American Type Culture Collection (Rockville, MD). Cells were maintained routinely at 37°C in a humidified 5% CO₂ incubator as monolayer cultures in α-MEM with ribonucleosides and deoxyribonucleosides growth media (provided by Media Department, Ontario Cancer Institute, Ontario, Canada) supplemented with 10% fetal bovine serum and the antibiotics penicillin and streptomycin.

Reagents and Chemicals. 9-AC was provided by Pharmacia Inc. (Albuquerque, NM). CPT was a gift of Dr. M. C. Wani (Research Triangle Institute, Research Triangle Park, NC). For all of the experiments, CPT and 9-AC were dissolved in dehydrated DMSO and stored at −70°C until used. high-performance liquid chromatography-grade methanol was purchased from VWR Scientific (Toronto, Ontario, Canada). All of the other chemicals were molecular biology grade and were purchased from Sigma (St. Louis, MO) unless otherwise specified.

The Kinetics of 9-AC in Cell Culture Media. Kinetic studies were performed in cell-free culture media at 37°C, 5% CO₂, at drug concentrations of 13.7 nM (5 ng/ml) and 137 nM (50 ng/ml). The media was left in the incubator overnight to allow the media to have the same condition as in cell cytotoxicity studies. Stock solutions (30 µl) of 9-AC at 13.7 µg/ml or 137 µg/ml were added to 30 ml of medium. A 1-ml sample was taken at 5, 10, 15, 20, 30, 45, and 60 min and at 2, 4, 8, 12, 24, 48, 72, and 240 h after the drug was added. A high-performance liquid chromatography method developed by Takimoto et al., (22) was used to extract and quantitate the 9-AC lactone, using CPT as the internal standard. The total 9-AC (lactone + carboxylate) concentration was extracted by a slight modification of the lactone extraction procedure. Media aliquots (0.1-ml) were acidified with 0.9 ml of 8.5% phosphoric acid and incubated at room temperature to allow for complete conversion of 9-AC carboxylate to 9-AC lactone. Then the samples were analyzed by the same method used for 9-AC lactone. The 9-AC peak was detected by using a Shimadzu RF-10A fluorescent detector at an excitation wavelength of 365 nm and an emission wavelength of 440 nm.

Cytotoxicity of 9-AC. The cytotoxicity of 9-AC was assessed by clonogenic assay (23, 24). Exponentially growing cells were resuspended in media, cell number was determined using an electronic counter, and 100–250 cells were inoculated in triplicate onto 60 15-mm dishes containing 5 ml of medium. After an overnight incubation, 5 µl of 9-AC stock solutions were added to the dishes to achieve final concentrations of 0, 0.27, 1.37, 2.74, 13.7, 27.4, 137, and 274 nM. After 4-, 8-, 12-, 24-, 48-, 72-, and 240-h exposures, medium was removed by aspiration and fresh medium was added to the dishes. Percentage of survival at each drug concentration with different exposure time was determined from the ratio of the number of the colonies in the drug-treated sample:the number in the control (DMSO vehicle-treated) sample.

Kinetic Analysis of the Hydrolysis of 9-AC Lactone. The concentration of intact lactone (C) versus time (t) date for the 9-AC were fitted to the equation below by the method of nonlinear least squares (25):

\[ C = a + b \exp(-k_1t) \]  

where the parameter \( k_1 \) is the pseudo first-order rate constant of hydrolysis of the lactone ring, from which \( t_{1/2} \) of hydrolysis can be calculated by dividing 0.693 by the rate constant, \( k_1 \). The parameter “a” corresponds to the concentration of the intact lactone form at equilibrium, and \( (a + b) \) equals the total intact lactone form of drug at \( t = 0 \) (when 9-AC was first added to media). Fitting of experimental data to the equation was accomplished using Scientist software (MicroMath Scientific Software, Salt Lake City, UT).

The observed equilibrium constant (\( k_{obs} \), for the conversion of the lactone to its corresponding ring opened species is calculated from \( \text{opened}_{eq}/(\text{closed}_{eq})_0 \) whereas the percentage opened was calculated from the fraction \( \text{opened}_{eq}/(\text{total})_{eq} \) (26). The value of \( k_{obs} \) was obtained from the average of three independent experiments.

The Effect-Concentration (E-C) Relationship. Fitting the experimental data to each of the following equations, we analyzed the relationship between cytotoxicity (E) and drug concentration (C).

sigmoidal \( E_{\text{max}} \) model:

\[ E = \frac{E_{\text{max}}C^n}{C^n + EC_{50}^n} \]  

where \( E \) is the survival percentage; \( C \) is the 9-AC concentration; \( E_{\text{max}} \) is the maximum drug effect; \( EC_{50} \) is the concentration that produces one-half of the maximum effect; and \( n \) is the Hill constant, which describes the shape of the curve.

In log-linear \( E-C \) model, \( a \) is a slope parameter and \( b \) is a constant. In the case in which the maximum effect was not achieved, the data were fitted to the log-linear model. If maximum effect was achieved, the data were fitted to Sigmoidal \( E_{\text{max}} \) model using nonlinear least squares regression Scientist software and values for \( IC_{50} \), \( IC_{50} \), \( n \), and \( E_{\text{max}} \) were determined.

The Effect-Concentration-Time (E-C-T) Relationship. The 9-AC AUC for each different exposure time was calculated using the LAGRAN V1.0B software (University of Alberta, Edmonton, Alberta, Canada). Then the relationship between the AUC of 9-AC lactone and the cytotoxicity of 9-AC was analyzed.

The relationship between drug concentration, exposure time, and cytotoxicity was fit to the following PD model (18):

log-linear \( E-C-T \) model:

\[ E = a^1 \log(C) + b \]  

In this model, \( C \), \( t \), \( n \), and \( k \) are the drug concentration, exposure time, drug concentration coefficient, and the drug exposure constant, respectively. \( n \) and \( k \) were determined from a plot of the isotoxic concentrations \( IC_{50} \) or \( IC_{50} \) versus exposure time using the least squares nonlinear regression using the Scientist software package (MicroMath Scientific Software). \( n < 1 \) indicates that the duration of exposure is more important than the
concentration. The minimum concentration \( C_{\text{min}} \) and time \( t_{\text{min}} \) to reach certain cytotoxicity were also calculated using the following equations:

\[
C^n \times t = k \quad \text{(D)}
\]

\[
C_{\text{min}} = (n \times k)^{\frac{1}{n+1}} \quad \text{(E)}
\]

\[
t_{\text{min}} = \frac{C_{\text{min}}}{n} \quad \text{(F)}
\]

\[
(C \times t)_{\text{min}} = C_{\text{min}} \times t_{\text{min}} \quad \text{(G)}
\]

**Cell Cycle Analysis.** Flow cytometry with propidium iodide staining was performed prior to and after 4- and 24-h drug exposures at drug concentrations of 13.7 nM (5 ng/ml) and 137 nM (50 ng/ml) by the procedure of Kastan et al. (27). Briefly, cells were harvested and fixed on ice in 80% ethanol for 1 h. Prior to flow cytometry, cells were washed with n-PBS (Ca\(^{2+}\)-Mg\(^{2+}\)-free-PBS) treated with 1 mg/ml RNase and stained with 50 μg/ml propidium iodide for at least 60 min. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson, Mansfield, MA). Data were gated on light scatter before the recording of a histogram composed of 10,000 cells. The percentage of cells in each phase of the cell cycle was quantified using the MOFlo software package (Verity Software House Inc., Topsham, ME). Each value shown in the results represents the mean ± SD of three or more independent experiments.

**RESULTS**

The **Kinetics of 9-AC.** Fig. 1 depicts changes in lactone concentration as a function of time for 137 nM 9-AC in media at 37°C and 5% CO\(_2\). The lactone concentration decreased rapidly in the first 2 h and reached equilibrium in media after a 6-h incubation. This equilibrium remained constant for the 10 days that this was studied. The total concentration of 9-AC (lactone + carboxylate) did not change significantly during the 240-h incubation (Fig. 1).

The concentration of 9-AC lactone \( C \) versus time \( t \) data were fitted to equation \( C = a + b \exp(-k_1 t) \) by the method of nonlinear least squares (Fig. 1). The pseudo first-order rate constant of hydrolysis of 137 nM 9-AC lactone was calculated to be 0.724 h\(^{-1}\). The result showed that the hydrolysis of 9-AC lactone proceeded with a \( t_{1/2} \) of approximately 57 min and achieved a final lactone:carboxylate equilibrium ratio of 35:65.

The observed equilibrium constant, \( k_{\text{obs}} \), for the conversion of the lactone to its corresponding ring opened species was calculated to be 1.857.

The kinetic profile of 13.7 nM 9-AC lactone in cell culture media was similar to that of 137 nM 9-AC, which indicated that the rate of this process was independent of drug concentration over the ranges examined in this study. Its pseudo first-order rate constant was 0.968 h\(^{-1}\), and the final lactone:carboxylate equilibrium ratio was 37:63. The observed equilibrium constant, \( k_{\text{obs}} \), was calculated to be 1.703.

**Cytotoxicity of 9-AC.** The survival percentages for the MCF-7, MGH-U1, and HT-29 cell lines at each 9-AC concentration with different exposure times all demonstrated minimal cytotoxicity until concentrations in excess of 3–5 nM were used. The data for cell line HT-29 is shown in Fig. 2. All of the three cell lines displayed some sensitivity to 9-AC (Table 1). When a maximum effect (100%) was approached, the curves for all of the three cell lines were sigmoidal in shape. The \( E_C^n \) data for the 3 cell lines were computer fitted to the log-linear and sigmoidal \( E_C^n \) models, and the estimates for \( n, E_{\text{max}}, IC_{50}, \) and \( IC_{90} \) were...
obtained (Fig. 3). Table 1 lists the fitted PD parameters of the three cell lines. The IC₅₀s for a 4-hour exposure were 23.28 nM (MGH-U1), 33.51 nM (MCF-7), and 126.51 nM (HT-29). The IC₅₀s for exposures of 24 h or longer were similar among the three cell lines. When looking at IC₉₀s, MGH-U1 was the most sensitive line and MCF-7 the most resistant. The range of concentration required to kill one log of cells in the different lines was 11.6–113.5 nM for a 24-h exposure and only 9.9–14.3 nM for a 72-h exposure. The cytotoxicity of 9-AC to the three cell lines increased with longer exposure time. However, the cytotoxicity was limited in the case of shorter duration of exposure (Fig. 2). When HT-29 and MCF-7 cells were exposed to 9-AC for less than 24 h, less than 1 log of cell killing occurred, even at the highest concentrations (274 nM). Increasing the exposure time from 4 h to 24 h decreased the IC₅₀ 6-fold for the MGH-U1, 9-fold for the MCF-7, and 21-fold for the HT-29 cell lines. An increase in the exposure time from 24 to 72 h only slightly decreased the IC₅₀ for the three cell lines; however, the concentration required to achieve 1 log of cell kill decreased from 11.6 to 9.9 nM, 113.5 nM to 14.3 nM, and 46.8 to 14.3 nM in MGH-U1, MCF-7, and HT-29, respectively. This indicates that exposure time beyond a threshold is a critical determinant of 9-AC cytotoxicity.

The cytotoxicity of 9-AC to the three cell lines also increased with higher drug concentrations. However, minimum cell killing was observed until concentrations greater than 2.7 nM were used. When 9-AC concentration was increased from 2.7 to 13.7 nM, great changes in cytotoxicity were observed for the MGH-U1 and HT-29 cell lines. Additional increases above 13.7 nM did not significantly change the cytotoxicity, even with longer exposures.

**PK/PD Modeling of 9-AC.** The AUC of 9-AC for each concentration at different exposure times was calculated using LAGRAN V1.0B software. The relationship between the AUC of 9-AC lactone and the cytotoxicity of 9-AC was then analyzed. Table 2 lists comparisons on the basis of similar AUC values for different time points and concentrations and the survival percentages of the three cell lines. With a similar AUC, the HT-29 cell line survival percentages ranged from 68 to 77%, 8 to 51%, and 3 to 27%. The same results were observed in the other two cell lines, which indicated no fixed relationship between AUC and survival percentage could be modeled.

The data from the multiple exposure time experiments for the three cell lines were then fitted to the PD model

\[ C_{\text{min}} \times t_{\text{min}} = k \]

Using this equation, we determined the PD parameters \( n \) and \( k \) from a plot of the isotoxic concentrations IC₅₀ or IC₉₀ versus time of exposure using least squares nonlinear regression fitting. \( C_{\text{min}} \times t_{\text{min}} \) and \( (C \times t)_{\text{min}} \) were then calculated from the \( n \) and \( k \) values. All of the parameters for the three cell lines are summarized in Table 3. For 50 or 90% of cell killing, the cytotoxicity-drug concentration-exposure time \((E-C-t)\) relationship could be fitted very well by this model with \( r^2 \) ranging from 0.90 to 0.99. To kill 90% of cells, \( n \) values ranged from 0.3 to 1.0

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**Table 1** 9-AC concentration-effect \((E-C)\) relationship parameters for three cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Exposure time (h)</th>
<th>IC₅₀ (nM)</th>
<th>IC₉₀ (nM)</th>
<th>( n )</th>
<th>( E_{\text{max}} ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGH-U1</td>
<td>4</td>
<td>23.3</td>
<td>NA</td>
<td>NA</td>
<td>97.4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>17.8</td>
<td>363.6</td>
<td>0.8</td>
<td>97.4</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>6.5</td>
<td>41.7</td>
<td>1.5</td>
<td>95.9</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.3</td>
<td>11.6</td>
<td>2.1</td>
<td>100.8</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>3.9</td>
<td>11.1</td>
<td>1.9</td>
<td>101.9</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>2.9</td>
<td>9.9</td>
<td>1.6</td>
<td>102.2</td>
</tr>
<tr>
<td>MCF-7</td>
<td>4</td>
<td>33.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>15.4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td></td>
<td>12</td>
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<td>NA</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>3.7</td>
<td>113.5</td>
<td>0.6</td>
<td>103.4</td>
</tr>
<tr>
<td></td>
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<td>1.6</td>
<td>23.0</td>
<td>0.8</td>
<td>99.6</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1.6</td>
<td>14.3</td>
<td>0.9</td>
<td>99.1</td>
</tr>
<tr>
<td>HT-29</td>
<td>4</td>
<td>126.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>58.5</td>
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<tr>
<td></td>
<td>12</td>
<td>22.0</td>
<td>NA</td>
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<td>NA</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.9</td>
<td>46.8</td>
<td>1.5</td>
<td>94.0</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.6</td>
<td>17.2</td>
<td>1.7</td>
<td>98.9</td>
</tr>
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<td>72</td>
<td>4.6</td>
<td>14.3</td>
<td>1.8</td>
<td>101.6</td>
</tr>
</tbody>
</table>

*90% inhibition was not achieved experimentally, and log-linear \( E-C \) model was used to fit data.

* NA, not applicable.
PK/PD Relationship of 9-AC

4-h exposure to 9-AC at both 13.7 nM and 137 nM had no demonstrable effects on cell cycle in any of the three cell lines. Consistent with the cytotoxicity studies, a longer-than-24-h exposure was necessary for all three of the cell lines (Table 4). To reach 90% cell killing, a longer-than-24-h exposure duration appeared to be more important. One log of cell killing occurred only when the cells were exposed for 24 h or longer. Extension of the duration of exposure beyond 24 h was more important for the more resistant lines than for the sensitive MGH-U1, in which little further increase in cytotoxicity occurred. These observations, which indicate that 9-AC-induced cytotoxicity required a threshold duration of drug exposure, is in accord with studies using other S-phase-specific antitumor agents such as hydroxyurea (32).

The AUC for 9-AC for different exposure points and their cytotoxicity for three cell lines is shown in Table 2. The AUC for 9-AC was calculated using LAGRA V1.0B software. Survival percentage of three cell lines was obtained from cytotoxicity studies.

<table>
<thead>
<tr>
<th>Initial 9-AC lactone conc. (nM)</th>
<th>Duration of exposure (h)</th>
<th>9-AC lactone AUC (nM-h)</th>
<th>% survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7</td>
<td>48</td>
<td>51.2</td>
<td>77.0</td>
</tr>
<tr>
<td>13.7</td>
<td>8</td>
<td>52.2</td>
<td>67.9</td>
</tr>
<tr>
<td>137</td>
<td>4</td>
<td>465.8</td>
<td>51.2</td>
</tr>
<tr>
<td>13.7</td>
<td>72</td>
<td>477.3</td>
<td>7.5</td>
</tr>
<tr>
<td>27.4</td>
<td>72</td>
<td>756.8</td>
<td>2.8</td>
</tr>
<tr>
<td>137</td>
<td>12</td>
<td>726.4</td>
<td>26.8</td>
</tr>
</tbody>
</table>

0.85 for all three cell lines, which indicated the relatively greater importance of exposure time to cytotoxicity. The $C_{\text{min}}$, $t_{\text{min}}$, and $(C \times t)_{\text{min}}$ values represented the critical exposure time and drug concentrations required for a certain drug effect and thereby provided a useful PD framework for additional in vitro studies. To reach 90% cell killing, a longer-than-24-h exposure was necessary for all three of the cell lines ($t_{\text{min}}$ = 50, 35, and 24 h, respectively), and $C_{\text{min}}$ was 25, 29, and 7 nM, respectively.

**Effects of 9-AC on Cell Cycle Progression.** The cell cycle effects of 9-AC were studied as a function of concentration and time (Table 4). Consistent with the cytotoxicity studies, 4-h exposure to 9-AC at both 13.7 nM and 137 nM had no demonstrable effects on cell cycle in any of the three cell lines. However, after 24-h exposures to 13.7 nM 9-AC, the HT-29 and MCF-7 cell lines arrested in G₂-M and remained there for 72 h after drug exposures. A G₂-M arrest was transient in the MGH-U1 cell line, in which cells re-entered into the cell cycle at 48 h and displayed DNA histograms similar to the controls at 72 h. After 24-h exposure to 137 nM 9-AC, all three cell lines accumulated in S phase. The MGH-U1 and MCF-7 cells remained in S phase at 72 h, whereas the HT-29 cells progressed through S phase and arrested in G₂-M 48 h after drug exposure and remained there at 72 h.

**DISCUSSION**

Because the active 9-AC lactone is unstable in aqueous solution, the kinetics of 9-AC lactone need to be studied to accurately determine the relationship between cytotoxicity and the AUC in vitro. The total concentration of 9-AC was also monitored to ensure that the concentration of 9-AC was constant throughout the process of cell killing. At equilibrium, the 9-AC lactone:9-AC carboxylate ratio in media was ~35:65, which was higher than that reported in human plasma (9.91; Ref. 28). Human serum albumin exhibits a 200-fold binding preference for the carboxylate relative to the lactone. The binding preference of the 9-AC carboxylate may accelerate the hydrolysis and shift the equilibrium favoring the formation of carboxylate, hence decreasing the 9-AC lactone percentage at equilibrium in human plasma.

Although there were differences in sensitivity of the 3 cell lines to 9-AC, these differences became less pronounced during longer exposure times. The MGH-U1 cell line had the shortest doubling time and was the most sensitive cell line among the three cell lines studied in our experiment (doubling times were 18, 28, and 36 h for MGH-U1, MCF-7, and HT-29, respectively). The CPTs are S-phase-specific agents and cell lines with a high S-phase fraction should be more sensitive to the drug (29, 30). Although we did see some differences in doubling times, there were no significant differences in S-phase fraction between the three lines. A threshold concentration of at least 2.7 nM 9-AC was required for any drug cytotoxicity, whereas a minimum of 7–29 nM was required for a log of cell kill. An increase in 9-AC concentration from 2.7 to 13.7 nM dramatically decreased the survival percentage for all of the three cell lines. A further increase in concentration above 13.7 nM only slightly decreased the survival fraction. These observations are in agreement with a previously reported preclinical study of 9-AC and suggest that maintenance of the 9-AC lactone within a critical threshold concentration range is important for efficacy against human cancers in vitro and in vivo (31). Although 9-AC cytotoxicity was related to concentration with a threshold being defined, the duration of exposure appeared to be more important. One log of cell killing occurred only when the cells were exposed for 24 h or longer. Extension of the duration of exposure beyond 24 h was more important for the more resistant lines than for the sensitive MGH-U1, in which little further increase in cytotoxicity occurred. These observations, which indicate that 9-AC-induced cytotoxicity required a threshold duration of drug exposure, is in accord with studies using other S-phase-specific antitumor agents such as hydroxyurea (32).

The PD model, $C^{\ast} \times t = k$, was able to fit the cytotoxicity results very well; to kill 90% of cells, the value of $n$ ranged from 0.3 to 0.85. An $n$ value of $<1$ suggests exposure time is more important than concentration, and these data, thus, indicate that exposure time is more important than concentration to achieve significant cytotoxicity. The flow cytometric analysis of cell cycle after drug exposure also supports the need for a minimum duration of drug exposure to achieve cytotoxic effects. These findings are consistent with those for other phase-dependent antitumor agents such as the antimetabolites, for which duration of exposure is more important than peak concentration (33–35). The time-dependency of 9-AC-induced toxicity seen in this study is also consistent with preclinical studies done of this drug. In a xenograft model, 9-AC by s.c. injection was toxic, and the implanted human cancer tumor regressed only partially (36). At identical dose levels, when 9-AC was given s.c. by depot injection with gradual release of the drug into the bloodstream, the treatment was without apparent toxicity and resulted in complete regression of implanted tumors.

Whether a schedule-specific therapeutic advantage can be obtained with the use of 9-AC is not yet determined. 9-AC schedules tested in Phase I studies reported to date are a 24-h infusion given weekly, a 72-h infusion given every 2–3 weeks, a 5–7-day infusion every 3 weeks, and a daily oral schedule (37–40). The dose-limiting toxicity in most studies is myelo-suppression with steady-state concentrations of 9-AC lactone of ~10, 2.6, and 0.5 nM at the maximally tolerated dose of 1.65 mg/m²/day i.v., 0.84 mg/m²/day i.v., and 0.84 mg/m²/day p.o. in the 24-h, 72-h, and daily oral schedules, respectively. Using a higher dose regimen, a 7-day infusion schedule in leukemic patients was able to achieve steady-state concentrations of 10

<table>
<thead>
<tr>
<th>Duration of exposure (h)</th>
<th>9-AC lactone AUC (nM-h)</th>
<th>% survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7</td>
<td>48</td>
<td>51.2</td>
</tr>
<tr>
<td>13.7</td>
<td>8</td>
<td>52.2</td>
</tr>
<tr>
<td>137</td>
<td>4</td>
<td>465.8</td>
</tr>
<tr>
<td>13.7</td>
<td>72</td>
<td>477.3</td>
</tr>
<tr>
<td>27.4</td>
<td>72</td>
<td>756.8</td>
</tr>
<tr>
<td>137</td>
<td>12</td>
<td>726.4</td>
</tr>
</tbody>
</table>
Table 3  PD parameters for 9-AC cytotoxicity to three cell lines

Parameters n, k, r² were determined by computer fitting the IC₅₀ or IC₉₀ data to the PD relationship Cⁿ × t = k, r² is the goodness of fit from the correlation of observed values to the predicted values. Cₘᵡ, tₘᵡ and (C × t)ₘᵡ were calculated from the n and k values as described in the “Materials and Methods” section.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Survival percentage</th>
<th>n</th>
<th>k</th>
<th>r²</th>
<th>Cₘᵡ (nm)</th>
<th>tₘᵡ (h)</th>
<th>(C × t)ₘᵡ (nm·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>50%</td>
<td>0.82</td>
<td>77.27</td>
<td>0.99</td>
<td>9.77</td>
<td>11.91</td>
<td>116.41</td>
</tr>
<tr>
<td>HT-29</td>
<td></td>
<td>0.75</td>
<td>146.55</td>
<td>0.93</td>
<td>14.67</td>
<td>19.56</td>
<td>286.95</td>
</tr>
<tr>
<td>MGH-U1</td>
<td></td>
<td>1.20</td>
<td>185.78</td>
<td>0.90</td>
<td>11.68</td>
<td>9.73</td>
<td>113.69</td>
</tr>
</tbody>
</table>

Survival percentage = 10%
MCF-7 0.51 257.63 0.98 25.59 49.59 1254.09
HT-29 0.85 613.76 0.95 29.44 34.64 1019.66
MGH-U1 0.30 42.85 0.93 7.13 23.77 169.46

Table 4  Cell cycle distributions after 24-h treatment in the MGH-U1, MCF-7, and HT-29 cell lines

Each value represents the mean ± SD of three or more independent experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGH-U1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.7 nM</td>
<td>37 ± 2</td>
<td>34 ± 1</td>
<td>29 ± 8</td>
<td>28 ± 0</td>
</tr>
<tr>
<td>137 nM</td>
<td>0 ± 0</td>
<td>95 ± 1</td>
<td>5 ± 1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.7 nM</td>
<td>48 ± 9</td>
<td>27 ± 13</td>
<td>25 ± 5</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>137 nM</td>
<td>15 ± 1</td>
<td>81 ± 6</td>
<td>4 ± 7</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>HT-29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.7 nM</td>
<td>53 ± 8</td>
<td>33 ± 5</td>
<td>14 ± 6</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>137 nM</td>
<td>3 ± 1</td>
<td>94 ± 3</td>
<td>3 ± 3</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

nm; however, severe, prolonged myelosuppression and mucositis were associated with this schedule. The studies we have performed suggest that the current strategy of using longer infusions of 9-AC is a reasoned approach to the clinical development of this drug. However, the use of infusions of longer than 24 h is leading to steady-state plasma concentrations at the maximally tolerated dose that are at, or below, the threshold for cytotoxicity in vitro. Thus, the use of shorter (i.e., 24 h) infusions of the drug, given repeatedly, may offer the greatest opportunity for clinical benefit.

REFERENCES
Pharmacological Determinants of 9-Aminocamptothecin Cytotoxicity

Mei-Li Li, Leora Horn, Patricia S. Firby, et al.


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