Application of in Vivo and in Vitro Pharmacokinetics for Physiologically Relevant Drug Exposure in a Human Tumor Clonogenic Cell Assay

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ABSTRACT

The biological half-lives and decay rate constants under the conditions of a human brain tumor clonogenic cell assay were determined for six clinically used anticancer agents. The agents studied were: 1,3-bis(2-chloroethyl)-1-nitrosourea; 3-(2-chloroethyl)-3-nitrosourea-2-deoxy-D-glucopyranoside; cis-diaminedichloroplatinum(II); 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone; 4-demethylepipodophylotoxin-D-thylidene glucoside; and 9-hydroxy-2-/V-methylellipticine. In vitro decay of all six drugs was found to be according to first order kinetics. The half-lives of two drugs, namely, 1,3-bis(2-chloroethyl)-1-nitrosourea and 3-(2-chloroethyl)-3-nitrosourea-2-deoxy-D-glucopyranoside under the human tumor clonogenic cell assay (HTCA) conditions were found to be similar to their terminal in vivo half-lives in humans. For the other drugs, however, there was a very large difference between their in vitro and in vivo pharmacokinetics. In the case of 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone, we observed about an 80-fold difference between its in vitro half-life of 40.76 h and its in vivo terminal half-life of 0.52 h. We describe the principles upon which these data can be used to design clinically more relevant in vitro drug exposure protocols in HTCAs. Since, generally, tumor cells are exposed to drugs in the HTCA either continuously or for a specified duration, e.g., 1 or 2 h, we computed the initial in vitro drug concentrations to which tumor cells should be exposed such that the resulting in vitro (c x t) after a 2-h or a continuous exposure will be within clinically achievable levels. The application of these in vitro and in vivo pharmacokinetic principles will provide for more physiological testing of patient tumor cell sensitivity to anticancer drugs in the HTCA, and is likely to result in lower rates of false positive responses in clinical trials using clonogenic cell assays.

INTRODUCTION

HTCAs1 (1-3) have been used to study tumor biology (4), to determine the sensitivities of tumors of patients to chemotherapeutic agents (5-10), and more recently to investigate the potential activity of new anticancer drugs (11). The optimum design of drug exposure protocols for performing clinically relevant drug sensitivity studies utilizing HTCAs, however, requires not only knowledge of the clinical pharmacology and pharmacokinetics of the agents being tested, but also information on drug stability and cell kill kinetics under the HTCA conditions. Such information is necessary to ensure that tumor cells are exposed to drugs at concentrations and for durations that are not only clinically relevant, but also are appropriate with the pharmacology, cell cycle phase specificity, and route of administration of the given drugs. Because data on intratumoral drug concentrations and drug decay kinetics are generally not available the clinical pharmacokinetic parameters most frequently used to determine drug exposures in clonogenic cell assays have been the drug peak plasma concentration, the drug plasma terminal half-life, and the drug plasma (c x t), representing the integral of drug plasma levels over time. However, in order to use these data to compute in vitro drug exposure times and drug concentrations such that the resulting in vitro (c x t) would be within clinically achievable values, one needs to know both the pharmacokinetic model that fits the dynamics of in vitro drug concentration changes over time and the drug decay rate constants under the conditions of exposure of the cells to the drug in the assay. Unfortunately, there is only little quantitative data (12, 13) available on the chemical and/or biological stability of chemotherapeutic agents under HTCA conditions.

In this study, we used a modification of a previously described bioassay (41, 42) to determine the in vitro stabilities of six antitumor agents, as reflected by their in vitro biological decay rate constants and half-lives under the conditions of a brain tumor clonogenic cell assay (14). The initial drug concentrations used in the bioassay were those concentrations which demonstrated a significant in vitro cell kill in a cytotoxicity prescreen using the 9L rat gliosarcoma cell line. The results show that while for the nitrosoureas in vitro half-lives under the clonogenic cell assay conditions were within the ranges of the terminal half-lives observed in clinical pharmacokinetic studies, for the other drugs there was a very significant difference between their in vivo and in vitro half-lives. Based on our data, we make suggestions as to in vitro drug concentrations in which (a) a 2-h treatment, and (b) a continuous exposure (greater than 5 in vitro half-lives) will result in drug exposures within clinically achievable levels. Using the principles described in this paper and the drug decay data we present, other in vitro drug concentrations and exposure durations can be computed with which more clinically relevant in vitro drug exposures can be achieved for different methods of clinical drug administration, e.g., intratraeral or i.p.

MATERIALS AND METHODS

9L Gliosarcoma. The 9L tumor cell line is an N-methylnitrosourea-induced gliosarcoma of the Fisher 344 rat. It produces solid brain tumors in rats within 2 weeks of intracerebral injection (15). The cells used for this study were maintained by weekly in vitro passages of monolayer cultures in MEM supplemented with EBS and 15% FCS. Human Glioma SF 126. The human glioma cell line SF 126 was originated in our laboratory from a fresh tumor biopsy obtained by craniotomy from a patient bearing a grade IV astrocytoma. The cells were cryopreserved in MEM containing 10% dimethyl sulfoxide and 20% FCS. Prior to use in these studies, thawed cells were washed twice with medium and cultured as monolayers in T75 flasks containing MEM, EBS, and 15% FCS. The cells were characterized by their positive reactivity to a monoclonal antibody, GE 2, which recognizes a glioma-associated antigen (16), using previously described methods (17).
All cultures used in the experiments were screened for and shown to be free of Mycoplasma contamination.

Drugs. The drugs studied were provided by the following suppliers: BCNU and Cis-Pt (Bristol Myers, Syracuse, NY); CHZ, VM 26, and AZQ (Drug Development Branch, National Cancer Institute, Bethesda, MD); HME (Sanofi-Labaz, Bordeaux, France, and kindly made available for these studies by Dr. Victor Levin).

Stock solutions were prepared by dissolving the weighed amounts of drug in different solvents, depending upon the solubility of the drug. Subsequent dilutions were made with Hanks’ balanced salt solution. All drug solutions were prepared just before use and were kept on ice prior to addition to the treatment flasks. The solvents used and the highest final concentrations in culture were as follows: BCNU (ethanol; 1:4,000), CHZ (dimethyl sulfoxide; 1:4,000), AZQ (N,N-dimethylacetamide; 1:10,000), HME acetate (H2O; 1:10,000); and Cis-Pt (MEM; 1:10,000). VM 26 was dissolved at 10 mg/ml in a solvent consisting of 150 mg benzyl alcohol, 300 mg N,N-dimethylacetamide, 2.5 g purified polyethylene glycol, 300 mg N,N-dimethylacetamide, sufficient maleic acid to achieve a pH of 5.1, and 4,715 g absolute ethanol. The final solvent concentration in culture was 1:100,000.

For each experiment a set of control flasks were set up which were treated with solvent only at the concentrations stated above. Cytotoxicity Prescreen. In order to determine the initial drug concentrations at which to expose cells for the bioassay, a cytotoxicity study was performed for each drug by using the 9L gliosarcoma cell line. Peak drug concentration as reported in clinical trials were used to determine the range of concentrations for this initial prescreening study which was performed as described below. Confluent monolayer cultures were trypsinized with 0.25% trypsin in 0.4% EDTA solution in Ca2+/Mg2+-free phosphate-buffered saline and seeded in triplicate T25 tissue culture flasks at cell numbers ranging between 50 and 5000 cells (depending on the expected cell kill). MEM supplemented with 20% FCS, and 5 x 10⁴ 2-h incubated (4000 rad) 9L cells were added, and the flasks were incubated at 37°C for 24 h in a humidified atmosphere of 5% CO2/air. Fifty μl of stock solution of each drug, prepared as previously described, were added to each set of flasks, the flasks were incubated for a further 24 h, and then rinsed and refed with fresh MEM containing 20% FCS. After 2 weeks at 37°C and 5% CO2/air, colonies were stained with a 3% crystal violet/methanol solution and counted under low power microscopy.

Biosay. The dose at which a 1-3-log clonogenic cell kill of the 9L tumor cell line was achieved in the prescreen was used as the initial concentration in the bioassay, which was performed as follows: a set of 4 T75 tissue culture flasks were set up, each containing 1.5 x 10⁶ SF 126 human glioma cells and 1.5 x 10⁵ heavily irradiated 9L cells in MEM containing 20% FCS. The flasks were incubated at 37°C and 5% CO2 in a humidified incubator for 24 h to allow the cells to attach to the culture flask surface. Stock drug solution was then added to each flask to yield the predetermined initial drug concentration. At various time points, triplicate 1.5-ml aliquots of the supernatant were removed and immediately filtered through 0.4-μm sterile cellulose acetate filters (Millipore, Bedford, MA). The first 0.5 ml was discarded and the remaining 1 ml was added to T25 tissue culture flasks, each containing the following: 10²–10⁵ 9L cells (depending upon the expected cell kill), 5 x 10⁴ heavily X-irradiated 9L cells, and MEM supplemented with 20% FCS. The time points at which the drug solutions were aliquoted were determined in pilot studies to be 0–60 min (BCNU, CHZ, and VM 26); 0–9 h (Cis-Pt); and 0–24 h (AZQ and HME). After 24 h at 37°C and 5% CO2, these second sets of flasks were rinsed twice, fresh MEM containing 20% FCS was added to them, and they were incubated as previously described. Colonies were stained and counted after 2 weeks.

Simultaneously, a dose-response study was performed as described in the prescreening experiments, but using ranges of drug concentrations which included the initial concentrations used in the bioassay described above.

Evaluation of Biosay Survival Curves. For each drug, the surviving fraction, S.F. (t) of 9L gliosarcoma cell lines at each treatment time point, was computed by using the equation

\[ S.F.(t) = \frac{CFE(t)}{CFE(0)} \]  

whereby CFE(t) is the colony-forming efficiency of cells treated with supernatant removed at time t and CFE(0) is the colony-forming efficiency of untreated control cells. CFE is defined as:

\[ CFE = \text{No. of colonies formed/no. of cells seeded} \]

The surviving fractions were plotted against the time points at which the supernatants were removed. Each S.F.(t) was subsequently converted to the corresponding drug concentration, C(t) by reading off from the simultaneously determined dose-response curve, the drug concentration necessary to achieve an equivalent reduction in survival in the 9L clonogenic system. The in vitro decay rate constant, k, was then calculated by using the equation for first order kinetics.

\[ -\frac{dc}{dt} = kC(0) \]

which upon integration gives:

\[ C(t) = C(0) \cdot e^{-kt} \]

where C(t) is the drug concentration remaining in the flask after time t and C(0) is the initial drug concentration.

Rearranging Equation D and taking logs gives

\[ k = \ln(C(0)/C(t))/t \]

and at

\[ t_n = 0.693/k \]

k was computed by linear regression analysis of the concentration-time curve obtained for each drug, and using Equation F, the half-life t_n of the drug under the treatment conditions was computed.

Computation of Concentrations for Clinically Relevant Drug Exposure. In order to determine the initial concentration to which tumor cells should be exposed such that the resulting in vitro (c x t) is equivalent to the clinically achievable (c x t), the trapezoidal approximation (18) was used:

\[ (c \times t) = \frac{1}{2}(C_0 + C_1)(t_1 - t_0) + \frac{1}{2}(C_1 + C_2)(t_2 - t_1) + \frac{1}{2}(C_n-1 + C_n)(t_n - t_{n-1}) \]

where C0, C1, C2... Cn are the drug concentrations remaining in culture after exposure times of t0, t1, t2... tn, respectively. Since we had previously determined the in vitro decay rate constant k, it is possible to compute the remaining drug concentration, C(t), in culture at any given time, t, by using Equation D.

Substituting the values in Equation G allows calculation of C(0), the initial drug concentration required to achieve the clinical (c x t) after in vitro exposure of the tumor cells for a given time, e.g., 2 h. For a continuous drug exposure (t = ∞), the approximation used for calculating C(0) is as follows:

\[ (c \times t) = C(0)/k \]

and rearranging gives

\[ C(0) = k \cdot (c \times t) \]

where k is the in vitro decay rate constant of the drug and C(0) is the initial concentration to which tumor cells would be exposed.

RESULTS

Cytotoxicity Prescreen. Fig. 1 illustrates the survival curve obtained with cis-platinum in the prescreening study performed on the 9L gliosarcoma cell line. This representative curve is typical of the survival curves for the other five drugs. The results obtained from the survival curves for all six drugs are summa-

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The amounts of drug weighed and the volume of solvent used to prepare the stock solutions were biologically the most stable under the in vitro clonogenic cell assay conditions, with decay rate constants of 0.008 h⁻¹, and half-lives of 80 h, 40.76 h, and 36.47 h, respectively. For HME an initial drop in concentration of approximately 30% was observed. In a previous study (17) using an in vivo pre-screening assay (see "Materials and Methods"). This curve is representative of the curves for the other five drugs studied.

<table>
<thead>
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<th>Drug</th>
<th>In vitro concentration (µM)</th>
<th>Surviving fraction of 9L cells</th>
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<tbody>
<tr>
<td>AZQ</td>
<td>1.0</td>
<td>1 x 10⁻⁴</td>
</tr>
<tr>
<td>Cis-Pt</td>
<td>3.0</td>
<td>3 x 10⁻²</td>
</tr>
<tr>
<td>VM 26</td>
<td>0.15</td>
<td>1 x 10⁻²</td>
</tr>
<tr>
<td>BCNU</td>
<td>30</td>
<td>4 x 10⁻²</td>
</tr>
<tr>
<td>CHZ</td>
<td>30</td>
<td>2.7 x 10⁻²</td>
</tr>
<tr>
<td>HME</td>
<td>10</td>
<td>2 x 10⁻³</td>
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The survival of 9L cells, depending upon the sensitivity of this cell line to the individual drugs.

**In Vitro Drug Decay Kinetics.** The biological in vitro decay curves of all six drugs are shown in Fig. 2 and were found to fit first order kinetics as described by Equations C and D. The half-lives, t₁/₂, and decay rate constants, k, were calculated for each drug by using linear regression analysis of the corresponding drug-decay curve. The results of these analyses are summarized in Table 2. Of the six drugs, HME, AZQ, and VM 26 were biologically the most stable under the in vitro clonogenic cell assay conditions, with decay rate constants of 0.008 h⁻¹, 0.017 h⁻¹, and half-lives of 80 h, 40.76 h, and 36.47 h, respectively. For HME an initial drop in concentration of approximately 30% was observed. In a previous study (17) using an HPLC technique, a similar rapid drop in HME concentration was observed in vitro and was shown to be the result of adsorption of the drug to the tissue culture flask and not due to actual drug decay. The relevant clinical pharmacokinetic data for all the drugs as published in various reports are given in Table 3.

Fig. 3 shows the results of the standard dose-response study that was performed simultaneously with the drug-decay study. The theoretical concentrations were computed, based upon the amounts of drug weighed and the volume of solvent used to make the stock solutions. The experimental drug concentrations were those that were read off from the standard dose-response curves, and were based upon the level of 9L clonogenic cell kill achieved at those drug concentrations. As Fig. 3 shows, there was a good correlation (r = 0.986) between the theoretical and the experimental drug concentrations determined by bioassay. The highest variation in the two concentrations was 30% for HME. As mentioned earlier, however, this discrepancy between the two HME concentrations was probably mainly a result of drug adsorption onto the surface of the tissue culture flask. Correcting for this adsorption, a variation of 9% was computed between theoretical and experimental HME concentrations.

**Drug Concentrations for Clinically Relevant in Vitro Drug Exposure.** Table 4 gives the computed drug concentrations required to achieve clinical (c x t) levels in the clonogenic cell assay after (a) a 2-h drug exposure, and (b) a continuous exposure. For BCNU and CHZ these two concentrations were very similar and were 25.2 and 24.6 µM (BCNU), and 30.2 and 29.1 µM (CHZ), respectively. In contrast, for HME, AZQ, Cis-Pt, and VM 26, there were very wide differences in the in vitro concentrations required to achieve clinically relevant (c x t) levels under the two exposure conditions stated above.

**DISCUSSION**

Several reports (5–10) have documented, both retrospectively and prospectively, that human tumor clonogenic cell assays are capable of predicting the response of an individual patient to cancer chemotherapy. For previously untreated ovarian cancer patients with good performance status, the assay has been reported to perform better than the best choice of chemotherapy by the physician (6, 9). Generally, however, these studies have shown that clinical response correlates better with in vitro resistance (greater than 90%) than for sensitivity (50–70%). Significant among the assay-related factors that could account for these relatively low levels of positive in vivo-in vitro correlation rates are the design of the in vitro drug exposure protocols, the method of determining in vitro sensitivity indices (19), and the criteria for establishing the cut-off index for in vitro sensitivity to be used in such correlations (20). Although Alberts et al. (21) have reviewed the application of pharmacological principles in HTCAs and have proposed the concept of a "cut off" concentration for determining in vitro drug sensitivity, it has not been possible, in most cases, to apply these principles in an optimized fashion to HTCAs. This is primarily because little (12, 13) and for some drugs, no quantitative data on in vitro drug-decay kinetics under HTCA conditions are available.

In the absence of such information, most drug sensitivity studies with the HTCA have been performed simply by exposing tumor cells to peak plasma drug concentrations, or a fraction (5–10%) of it, continuously or for 1–2 h.

In this report, a bioassay was used to investigate the pharmacokinetics of six clinically used anticancer agents under the conditions of a human brain tumor clonogenic cell assay. For all the drugs investigated, in vitro decay was according to first order kinetics. Similar first order kinetics have been reported for the in vitro decay of both cis-platinum (22) and BCNU (23) in human plasma. Under the HTCA conditions used in this present study, the two nitrosoureas, BCNU and CHZ, demonstrated in vitro half-lives of 0.54 and 0.52 h, respectively. These values are relatively close to their respective in vivo terminal half-lives of 0.36 and 0.4 h, as determined in human clinical phase I trials (23, 24). Furthermore, for these two drugs, because of their relatively high in vitro decay-rate constants of 1.28 h⁻¹ (BCNU) and 1.33 h⁻¹ (CHZ) and correspondingly high in vitro survival of 9L cells, depending upon the sensitivity of this cell line to the individual drugs.

**Table 1** Prescreen cytotoxicity data on 9L gliosarcoma cell line

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short in vitro half-lives, in vitro drug concentrations required to achieve clinically relevant (c x t) levels were similar for both a 2-h and a continuous drug exposure. In contrast, for AZQ, Cis-Pt, VM 26, and HME, we showed in this study that wide differences existed between their decay kinetics in the HTCA and their published human clinical plasma pharmacokinetics (17, 23–29). The in vitro half-lives of all four drugs were much longer than their in vivo terminal half-lives. For example, Curt et al. (26) reported an in vivo plasma terminal half-life of 0.52 h for AZQ compared with a half-life of 40.76 h we measured under our HTCA conditions. The terminal plasma disappearance rate of AZQ is therefore, almost 80 times faster than the biological decay rate of the drug in the HTCA. This long half-life of AZQ determined by bioassay may reflect, in part, the
Thus, for AZQ, HME, Cis-Pt, VM 26, and other drugs. Consequently, for AZQ, HME, Cis-Pt, VM 26, and other drugs to the first order decay kinetics we observed for AZQ and the resulting decay curves will be bi- or multiphasic, in contrast to that of the parent drug, and decay of the drug intermediate is the rate-limiting step in the decay of the parent drug. Theoretically, in such situations, as of course is also the case for a drug whose cytotoxic action is due to the parent drug directly, a bioassay will provide for a true measure of the parent drug decay. Furthermore, first order kinetics, as we observed for all six drugs, strongly suggests the existence of only one major cytotoxic species. If more than one major cytotoxic species were to be present, then because of the expected different decay rate constants of these species, a bi- or multiphasic decay curve would be obtained.

The good correlation, $r = 0.986$, we observed between the theoretical and the bioassay-determined drug concentrations suggests that for the six drugs, the bioassay as described here allows for reasonably accurate determination of drug concentrations, and may be used in the absence of more specific assays requiring HPLC and other more complex techniques. However, a study comparing drug decay as measured by HPLC and by bioassay needs to be performed to confirm this for individual drugs.

Because of the differences in complexity of different HTCA techniques, differences are likely to exist in drug-decay kinetics under the different assay conditions. Such assay-specific variation in in vitro drug pharmacokinetics may require that drug decay be determined for each HTCA technique and the values obtained be used to optimize drug exposure in that assay. This problem may become less significant as the HTCAs become more sophisticated.

INDs pose an additional problem since there are usually no human pharmacokinetic data available for these agents at the time of initial screening. In the National Cancer Institute-sponsored multilaboratory study on the application of the HTCA to IND screening (11), tumor cells were exposed to drugs continuously at a single arbitrary concentration of 10 mg/ml. Active agents were then subjected to a dose-response study in order to better define their 70% infective dose values. This approach did not address any potential differences in the in vitro stability of the drugs. It may be advantageous even at this early stage of IND screening, using a simple bioassay such as we describe here, to determine the in vitro decay kinetics of agents that may be going into clinical trials. The data thus obtained could be used with clinical pharmacokinetic information that will be acquired during phase I clinical trials of active agents to better design further clinical trials of the agents with HTCA.
In this present report, the clinical pharmacokinetic data used to perform the computations were all derived from published clinical phases I and II trials, in which drug administration was done i.v. The peak plasma drug levels, the achievable \((c \times t)\), to perform the computations were all derived from published data and to Karin McDougall for typing of this manuscript.

PHARMACOKINETICS FOR DRUG EXPOSURE IN HTCA


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