Influence of Schedule on Alkylating Agent Cytotoxicity in Vitro and in Vivo

Beverly A. Teicher, Sylvia A. Holden, J. Paul Eder, Terrence W. Brann, Steven M. Jones, and Emil Frei III

Dana-Farber Cancer Institute, Boston, Massachusetts 02115

ABSTRACT

High dose, multiple alkylating agent chemotherapy is being employed in conjunction with autologous marrow transplantation in the clinic. We have investigated the scheduling of several alkylating drugs in an effort to optimize their antitumor effects. In vitro modeling of “continuous” (up to 72 h) versus “bolus” (1 h) exposure in MCF-7 cells showed that for 2,2',2"-triethylthiophosphoramide (thiotEPA), cis-diaminedichloroplatinum(II) (CDDP), 4-hydroxyresorcylic phosphamide, carboplatin, and 1-phenylalanine mustard (l-PAM) “continuous” exposure yields essentially the same killing kinetics as “bolus” exposure. For N,N,N"-bis(2-chloroethyl)-N-nitrosourea (BCNU), however, even with fresh drug additions every 30 min, “bolus” exposure produced superior cytotoxicity. In vivo modeling of “continuous” (three i.p. injections over 9 h) versus “bolus” (single dose) administration of the alkylating agents cyclophosphamide, BCNU, thiotEPA, melphalan, CDDP, and carboplatin was conducted in mice bearing EMT6 tumors, and tumor cell killing as measured by tumor cell survival in vitro was compared with killing of bone marrow (CFU-GM) measured in culture as a representative sensitive normal tissue. With cyclophosphamide there was a considerable increase in the therapeutic index (killing of tumor cells/killing of CFU-GMs) when the same total dose of drug was administered in multiple injections versus a single injection. For BCNU and thiotEPA, smaller increases in therapeutic index were observed. With l-PAM and CDDP, some advantage to multiple versus single dose administration was observed, and for carboplatin a decrease in the therapeutic index was seen. In conclusion, for all six alkylating agents examined, the multiple dose schedule was at least as effective against the tumor as the single dose schedule at all dose levels.

INTRODUCTION

In developing effective chemotherapeutic regimens employing very high drug doses, proper scheduling and sequencing may be critical (1-8). Although the importance of scheduling has been addressed extensively and is recognized as critical to therapeutic outcome in the case of many nonalkylating agents such as ara-C,7 which is cell cycle specific, this issue has not often been addressed for alkylating agents. The importance of scheduling with ara-C was demonstrated in murine L1210 by Skipper and colleagues, who showed that scheduling small doses of ara-C administered every 3 h for eight injections repeated every fourth day had a greater therapeutic effect than larger daily doses of drug (9, 10). Our interest in this question is a result of our clinical work with intensification, that is high dose alkylating agents with autologous bone marrow transplant. We have previously examined the issue of alkylating agent dose in tumor model systems (11, 12). In this report we have focused on the scheduling (single versus multiple doses) of CDDP, carboplatin, BCNU, CTX, thiotEPA, and l-PAM in vitro in the MCF-7 human breast carcinoma line and in vivo in the EMT6 mouse mammary carcinoma as single agents with respect to tumor cell kill and bone marrow (CFU-GM) kill examining both synergy and therapeutic index.

MATERIALS AND METHODS

Drugs. BCNU, CTX, and thiotEPA were obtained from the Dana-Farber Cancer Institute pharmacy. l-PAM was purchased as the pure powder from Sigma Chemical Co. (St. Louis, MO). CDDP and carboplatin were gifts from Johnson Matthey (Malvern, PA). For in vivo testing, drugs were prepared freshly just prior to use with PBS as the final diluent (13).

Cell Line. MCF-7 is a human adenocarcinoma of the breast, developed by Dr. M. Rich of the Michigan Cancer Foundation. This line is estrogen receptor-positive and retains certain characteristics of breast adenocarcinoma. MCF-7 has been used as a model for in vitro studies of breast carcinoma (14, 15). MCF-7 human breast carcinoma cells grow as monolayers in Dulbecco’s minimal essential medium supplemented with antibiotics, t-glutamine and 10% fetal bovine serum. These cell lines have a plating efficiency of 25-40%.

Survival Curves. MCF-7 cells in exponential growth were treated with various doses of the drugs for various periods of time in media without serum. The drugs were replenished after various exposure times, depending upon the stability of the agent (as shown in Table 1), by removing the drug-containing media, washing once with PBS, then replacing with fresh media and drug. At the termination of the exposure time, cells were washed with PBS and suspended by treatment with 0.25% trypsin. The cells were plated in duplicate at three dilutions for colony formation. After 2 weeks the colonies were visualized by staining with crystal violet, and colonies of 50 cells or greater were counted. The results were expressed as surviving fraction of treated cells compared to vehicle-treated control cells.

Tumor Line. The EMT6 murine mammary carcinoma is an in vivo in vitro tumor system (16-19). The EMT6 tumor was carried in BALB/c mice (Taconic Farms, Germantown, NY). For the experiments, 2 \times 10^6 tumor cells prepared from a brei of several stock tumors were implanted i.m. into the legs of BALB/c mice 8-10 weeks of age.

Tumor Excision Assay. For each experiment, two tumors were implanted per mouse and there were two animals at each dosage level; therefore, four tumors were pooled at each point. The determination of tumor cell kill, per experiment, was based on an assay of pooled tumor cells from four tumors. When the tumors were approximately 100 mm^2 in volume (about 1 week after tumor cell implantation) the drugs were administered as single doses by i.p. injection (0.2 ml) or as three doses by i.p. injection at 4.5-h intervals over 9 h. Mice were sacrificed 24 h after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage and then soaked in 95% ethanol. The tumors were excised and single cell suspensions were prepared as described previously (20). The untreated tumor cell suspensions had a plating efficiency of 8-12%. Results are expressed as the surviving fraction \pm SE of cells forming colonies of 50 cells or more in 60-mm dishes from treated groups compared to untreated controls from three independent experiments.

Bone Marrow Toxicity. Bone marrow was taken from the same animals used for the tumor excision assay. A pool of marrow from the femurs of two animals was obtained by gently flushing the marrow through a 23-gauge needle and CFU-GM assay was carried out as described previously (20). Colonies of at least 50 cells were scored on an Acculite colony counter (Fisher Scientific, Springfield, NJ). The results from three experiments, in which each group was measured at three cell concentrations in duplicate, were averaged. The results are

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2 The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; CDDP, cis-diaminedichloroplatinum(II) (cisplatin); BCNU, N,N,N"-bis(2-chloroethyl)-N-nitrosourea; l-PAM, melphalan, l-phenylalanine mustard; thiotEPA, N,N,N"-tri-ethyleneethiophosphoramide; CTX, cyclophosphamide; 4-HC, 4-hydroxyresorcylic phosphamide; PBS, phosphate buffered saline; IC_50, 90% inhibitory concentration; CFU-GM, granulocyte-macrophage colony-forming units.

5994
SCHEDULING OF ALKYLATING AGENTS

Table 1  Concentration × exposure time values for MCF-7 cells exposed to various alkylating agents

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration × time (μM × h)</th>
<th>exposure time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ThiotEPA</td>
<td>185</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td></td>
<td>166</td>
</tr>
<tr>
<td>CDDP</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>640</td>
<td>600</td>
</tr>
<tr>
<td>4-HC</td>
<td>44</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>BCNU</td>
<td>230</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
</tr>
<tr>
<td>L-PAM</td>
<td>30</td>
<td>40</td>
</tr>
</tbody>
</table>

* Drug replenished every 2 h.
* Drug replenished every 12 h.
* Drug replenished every 30 min.
* Drug replenished every 4 h.

Data shown for survival level of 1 log of cell kill.

RESULTS

Alkylating agents are generally short-lived drugs with complex pharmacology (23). Optimum scheduling of these drugs would maximize tumor cell kill without an equivalent increase in normal tissue toxicity. We have in vitro and in vivo models to compare the effect of exposure to a high dose of drug over a short-time interval versus over a longer time period by renewing the drug in vitro or administering multiple injections of drug in vivo. In Table 1, MCF-7 human breast carcinoma cells were exposed to several alkylating agents over a concentration range for 1 h or longer time periods and the product of the concentration and exposure time (C × T) which produced 1 log of tumor cell kill was calculated. For exposure times longer than 1 h, the drug concentration in the media was replenished by removing the drug-containing media and replacing it with fresh drug-containing media at the intervals shown. The drugs thiotEPA, CDDP, 4-HC, carboplatin, and L-PAM maintained their ability to kill tumor cells in the “continuous” exposure model as compared to the 1-h exposure data. However, with BCNU, although the drug was replenished at 30-min intervals some loss of effectiveness was seen at the longer exposure times compared to the 1-h exposure, as indicated by the increase in the concentration × time values.

The tumor cell survival (in vivo/in vitro) assay allows quantitative assessment of the impact of chemotherapy on both tumor and bone marrow clonogenic cell survival into the intensification range which is achieved in the clinic with autologous bone marrow transplantation. Continuous i.v. administration of alkylating agents in the mouse is technically difficult, therefore, single doses of an alkylating agent across a dosage range were compared with three doses given at 4.5-h intervals to the same total dose.

The survival of EMT6 tumor cells from tumors and bone marrow (CFU-GM) from the same animals treated with CTX as a single injection or as three injections given at 4.5-h intervals over 9 h to the same total dose is shown in Fig. 1. The killing of both tumor and bone marrow cells with CTX increases with increasing drug dose. The slopes of the survival curves for the killing of tumor cells are steeper than those for the killing of bone marrow cells. Administering CTX on the multiple injection schedule resulted in a 5- to 6-fold increase in tumor cell kill over the dosage range. On the other hand, the multiple injection regimen for CTX was 3- to 6-fold less toxic to the bone marrow than a single injection of the same total dose of the drug. Overall, administration of CTX in multiple injections increased the difference between tumor cell kill and bone marrow toxicity from 5- to 90-fold at 150 mg/kg of drug and from 40-fold to 1000-fold at 500 mg/kg of drug.

BCNU also showed an increase in tumor cell kill when administered on a multiple injection schedule compared to a single dose administration (Fig. 1). There was a 10- to 12-fold increase in tumor cell kill with BCNU given as multiple injections. The toxicity of BCNU to the bone marrow, however, was also increased on the multiple injection schedule. In the bone marrow there was a 4- to 5-fold increase in toxicity with three injections of the drug compared to a single dose. The difference between the kill of tumor cells as compared to bone marrow cells increased from 12- to 40-fold at 100 mg/kg of BCNU and from 30- to 100-fold at 200 mg/kg of BCNU when the drug was given as three injections compared to a single injection of the same total dose of the drug.

In the lower dosage range, thiotEPA on a single dose schedule was more toxic to bone marrow CFU-GM than to tumor cells (Fig. 2). When thiotEPA was administered on the multiple-dose schedule, there was an increase in tumor cell kill of 7- to 10-fold over the dosage range examined. At the lowest dose of thiotEPA (10 mg/kg) single dose and multiple dose schedules produced the same level of bone marrow toxicity; however, with 30 mg/kg of thiotEPA on the multiple dose schedule there was about a 5-fold greater kill of bone marrow CFU-GM compared to the single dose regimen. On the multiple dose schedule thiotEPA was always more toxic to tumor cells than toward bone marrow cells to an extent which ranged from 2- to 13-fold over the dosage levels examined.

L-PAM also increased in toxicity toward both tumor and bone marrow cells when administered as three injections as compared to a single dose administration (Fig. 2). The increase in tumor cell kill by L-PAM ranged from 8- to 15-fold over the same dosage range. Therefore, although administering L-PAM on a multiple dose schedule resulted in greater tumor cell kill for the same total dose of drug compared to single dose treatment, there was an equivalent increase in bone marrow toxicity. Therefore, no increase in therapeutic index for L-PAM was obtained by this strategy.

With CDDP on a single dose or multiple injection protocol there was an increasing differential between tumor cell kill and bone marrow kill with increasing dose of the drug (Fig. 3). Overall, administration of CDDP on the multiple injection protocol increased tumor kill by 3- to 4-fold compared to the tumor cell kill obtained with single dose administration of the drug. On the other hand, there was an increase in the slope of the bone marrow toxicity curve with CDDP on the multiple dose schedule compared to single dose administration. This resulted in an increase from 2- to 5-fold in bone marrow toxicity over the dosage range tested for the multiple injection regimen compared to single dose treatment. Overall, administration of CDDP in multiple injections resulted in only a slight increase in therapeutic index at higher doses of the drug, using bone marrow as a representative normal tissue.

Carboplatin, when administered in a single dose protocol, was 20- to 40-fold more toxic toward tumor cells than toward bone marrow CFU-GM (Fig. 3). Administering carboplatin on a multiple dose schedule did not significantly alter the toxicity of the drug to tumor cells. As with CDDP, when carboplatin was given on the multiple dose schedule there was a change in the slope of the bone marrow survival curve such that although...
Fig. 1. Comparison of survival of EMT6 tumor cells and bone marrow (CFU-GM) cells treated in vivo with single doses or with three doses at 4.5-h intervals to the total dose shown of CTX (left) or BCNU (right). Single drug dose for tumor cells (●) and the corresponding bone marrow (○); multiple drug injections to the total dose shown for tumor cells (●) and the corresponding bone marrow (○). Results are presented as the mean of three independent determinations; bars, SE.

Fig. 2. Comparison of survival of EMT6 tumor cells and bone marrow (CFU-GM) cells treated in vivo with single doses or with three doses at 4.5-h intervals to the total dose shown of thioTEPA (left) or l-PAM (right). Single drug dose for tumor cells (●) and the corresponding bone marrow (○); multiple drug injections to the total dose shown for tumor cells (●) and the corresponding bone marrow (○). Results are presented as the mean of three independent determinations; bars, SE.

at lower doses there was no difference between bone marrow toxicity for carboplatin on either the single or multiple dose schedules, at the highest dose of drug tested, carboplatin was 5-fold more toxic to bone marrow when administered as multiple injections compared to the single dose schedule. Overall, therefore, with carboplatin at lower doses there was no difference between tumor cell kill and bone marrow toxicity when the drug was given as a single treatment or by multiple injections; however, at the highest drug dose, there was a decrease in therapeutic index when carboplatin was given in multiple injections.

DISCUSSION

Alkylating agents are one of the most important classes of antitumor drugs (11, 12). Chemically, alkylating agents are quite heterogeneous and undergo complex activating and deactivating metabolism, depending on the specific molecule involved (23). The antitumor actions of these agents alone and in combination has been shown to vary with scheduling and sequencing of the drugs (2–8).

Because many alkylating agents are relatively unstable in aqueous solution, in an attempt to model in vitro the effect of a “bolus” (i.e., a high dose/short time exposure to the drug) compared to a “continuous infusion” (i.e., a lower dose/longer time exposure to drug) it was necessary to replenish the drugs over the exposure time period. The schedule for replenishing the drugs over the cells in the studies was determined by the known half-lives for the agents in aqueous solution. The removal and replacement of media necessarily resulted in some stress to the cells; despite this, however, for five out of six...
alkylating agents tested the concentration x time value remained constant, indicating that for those five drugs (thiotEPA, CDDP, 4-HC, carboplatin, and l-PAM) continuous exposure to a lower drug dose (if active drug levels remain constant) should produce tumor cell kill equivalent to a high dose/short exposure. It may be concluded from these in vitro studies that in general continuous exposure to alkylating agents produces an effect comparable to single dose exposure when corrected for area under the curve. However, while results from this monolayer culture model are consistent with no compromise in cytotoxicity if drugs are given as a continuous infusion, it remains possible that a different situation may pertain in vivo in a solid tumor mass.

The EMT6 mouse mammary carcinoma line grows as a solid tumor in mice which metastasizes only very rarely and as a cell line in culture (16–18). This in vivo-in vitro capability allows the response of solid EMT6 tumors to various treatments in vivo to be assessed quantitatively in vitro as tumor cell kill (17, 18). The correlation of the tumor cell survival assay and tumor growth delay assay for the EMT6 tumor has been described in greatest detail for radiation treatment (17, 18).

The tumor excision assay allows quantitation of tumor cell kill in tumors exposed to drugs in vivo. Three drug injections over 9 h were used to model “continuous” exposure compared to a single “bolus” injection of the drugs. Bone marrow CFU-GM was used as a representative normal tissue to make comparisons between killing of normal versus malignant cells. With the “continuous” schedule, a very positive effect was observed with multiple doses of CTX. There was greater tumor cell killing and less bone marrow cytotoxicity with CTX using the “continuous” exposure model. A very positive effect was also obtained with BCNU given in multiple injections since the increase in tumor cell killing with BCNU was greater than the concomitant increase in bone marrow toxicity, resulting in a therapeutic gain. The multiple injection schedule resulted in some gain in therapeutic efficacy with thiotEPA for the same reason. For the three other alkylating agents examined (l-PAM, CDDP, and carboplatin), there appeared to be no significant difference in therapeutic effect between administering the total dose of the drug as a single “bolus” injection or as multiple doses over 9 h. With carboplatin at very high doses the use of multiple injections appeared to result in some decrease in the therapeutic index.

In the bone marrow transplant setting in humans, CTX can be administered at dosage levels about 6- to 12-fold higher than in standard treatment regimens. BCNU can be administered at threefold greater than normal doses with bone marrow transplantation. The doses of thiotEPA and l-PAM used in the transplant setting are about 10-fold and about 5-fold higher than that used in standard regimens. The platinum complexes are more difficult to dose escalate; CDDP is used at about 1.6-fold of the normal dose and carboplatin is used at about 2.5-fold of the normal dose in the transplant setting. In the clinic, patients are given treatments which support and protect normal tissues such as the kidney and GI system in addition to the bone marrow transplantation. In these studies, we have extended the tumor cell survival curves to doses which are lethal to the animals and produce toxicities in addition to bone marrow toxicity. The dose limitation in these studies was that the animals survive for 24-h post-drug administration. The maximum dose of CTX in this study is 5- to 6-fold above the normal therapeutic murine dose. With BCNU the highest dose in this study is 10- to 15-fold greater than normal dosage range. ThiotEPA and l-PAM could only be dose escalated 3- to 4-fold and 2- to 3-fold in these EMT6 tumor-bearing mice, respectively. Finally, CDDP was administered at doses up to about 3- to 4-fold greater than normal and carboplatin was administered at doses up to about 10-fold greater than normal in this study. Therefore, with respect to dose escalation as described by a fold of the normal dose as compared between mouse and man, we have achieved comparable dose levels in mice with CTX and l-PAM. We have underescalated thiotEPA, slightly overescalated CDDP and substantially over escalated BCNU and carboplatin.

Most important was that for all of the six alkylating agents examined, the multiple dose schedule was at least as effective against the EMT6 tumor as the single dose schedule at all dose levels. Indeed, for CTX, BCNU, thiotEPA, l-PAM, and

![Fig. 3. Comparison of survival of EMT6 tumor cells and bone marrow (CFU-GM) cells treated in vivo with single doses or with three doses at 4.5-h intervals to the total dose shown of CDDP (left) or carboplatin (right). Single drug dose for tumor cells (●) and the corresponding bone marrow (○); multiple drug injections to the total dose shown for tumor cells (●) and the corresponding bone marrow (○). Results are presented as the mean of three independent determinations; bars, SE.](cancerres.aacrjournals.org)
CDDP, there was a 3–12-fold greater cytotoxicity for multiple doses as compared to single doses. These data along with the in vitro data indicate that continuous administration of single alkylating agents could be at least as effective as bolus administration of the same total dose.

These results contrast in some aspects with those obtained in the L1210 leukemia (24, 25). Schabel et al. (24) found that a single dose of 250 mg/kg of CTX administered i.p. on Day 1 following i.p. implantation of 10^6 L1210 cells on Day 0 was as therapeutically effective as 16 daily i.p. injections of 90 mg/kg of CTX (total dose 1350 mg/kg, an ultimately lethal dose of CTX). Similarly, with BCNU, single injection therapy on Day 1 at the maximum tolerated dose of BCNU results in a 100% cure rate. However, daily treatment for 16 days of a lower dose of BCNU but totalling far more drug in cumulative dose, cures only 20% of the mice and is ultimately lethal (25). The L1210 leukemia is a very rapidly growing ascites tumor which spreads throughout the body including the brain. The rapid increase in tumor cell number with time clearly favors an early, high dose therapeutic attack in order to achieve cure. In this study, established solid tumors were present at the initiation of treatment which was administered either as a single dose or as the same dose over a 9-h period. Tumor cell growth kinetics is not the main issue here. Drug metabolism, both activation and inactivation, and penetration of the tumor by active drug are larger factors. It is most likely that a low dose therapy over 16 days would be less effective therapeutically in the EMT6 tumor than a single high dose drug treatment as was the case in the L1210 leukemia.

Combination chemotherapy has been a critical strategy for achieving the curative treatment of clinical cancer with conventional doses. The same seems quite likely to be true for intensification autologous bone marrow transplantation programs. Many additive and synergistic treatment combinations derived from experimental studies require that the target cells see the agents used in combination simultaneously.

While we tend to focus on the cancer cell with respect to determinants of cytotoxicity, physiological structure in a solid tumor plays a significant role. Many solid tumors are poorly vascularized and thus access of chemotherapeutic agents to tumor cells distal from the vasculature may be a problem. Similarly, hypoxia, low growth fraction, pH, and multiple other characteristics of solid tumors and the surrounding environment may impact on the schedules of alkylating agents. This is an important area for current and future research.

REFERENCES

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