

Preclinical Studies and Clinical Correlation of the Effect of Alkylating Dose¹

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ABSTRACT

Dose-response studies were performed with the alkylating agents [nitrogen mustard, *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea, melphalan, cisplatin (CDDP), 4-hydroperoxycyclophosphamide (4-HC), and trimethyleneiminethiophosphoramidate] in both the MCF-7 human breast carcinoma cell line and the EMT6 and FSAiC murine tumor lines. Increasing selection pressure with the alkylating agents CDDP, melphalan, and 4-HC *in vitro* produced low levels (6.5- to 9-fold) of drug resistance, despite an intensive and prolonged treatment program. The MCF-7 sublines made resistant to CDDP and 4-HC did not exhibit cross-resistance to other alkylating agents; however, the MCF-7 subline resistant to melphalan was partially cross-resistant to nitrogen mustard, 4-HC, and CDDP. A log-linear relationship was maintained between surviving fraction of MCF-7 cells in culture and drug concentration with alkylating agents, whereas for nonalkylating agents the survival curves tended to plateau at high drug concentrations. Log-linear tumor cell kill was also obtained over a wide dosage range with several alkylating agents in murine tumors treated *in vivo*. Tumor cell survival assay by colony formation indicated the continuing importance of dose in the action of the drugs even at high levels of tumor cell kill. With some agents, there was a difference between the slopes of the tumor cell killing curves *in vivo* as compared to *in vitro*. Cyclophosphamide was far more potent *in vitro* (4-HC) than *in vivo* (cyclophosphamide). Trimethyleneiminethiophosphoramidate and *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea were both more potent *in vivo* than *in vitro*. These differences may be explained by the various metabolic patterns of these drugs. Dose of alkylating agents is clearly a crucial variable particularly where multilog tumor cell kill is the goal, and in this regard, the effect of drug dose on the tumoricidal action of the alkylating agents is substantially greater than for nonalkylating agents.

INTRODUCTION

The alkylating agents represent one of the most important classes of antitumor drugs (1). There has been increased interest in these agents in recent years partly because dose-limiting toxicity is often myelosuppression, and thus they are ideal agents for high-dose bone marrow transplantation regimens (2-7). Chemically, alkylating agents are quite heterogeneous, leading to different mechanisms of resistance to specific agents within the class and, generally, to a lack of cross-resistance among these drugs (8-10). While dose response for alkylating agents is generally considered to be steep, the dose or concentration required to achieve kill of many logs of tumor cells necessary for cure is not well defined against human tumor cells *in vitro* or in experimental solid tumor systems *in vivo*. Moreover, the implications of drug resistance on the effect of dose on alkylating agent cytotoxicity has not been previously studied.

As a class, alkylating agents are unique among the available antineoplastic drugs for several reasons. First, most anticancer drugs are more effective against proliferating than against nonproliferating cells. Many anticancer drugs are most effective against cells in one phase of the cell cycle. For example,

ara-C,² hydroxyurea, MTX, and 6-mercaptopurine have been described as S phase specific. VCR and vinblastine have been described as M phase specific (11). Alkylating drugs and anti-tumor antibiotics, on the other hand, are cell cycle phase nonspecific in cytotoxic action but are generally more toxic toward proliferating cells and show enhanced cytotoxicity toward cells in a specific phase of the cell cycle (12). Second, drugs reach tumor cells by diffusion from tumor blood vessels. The ability of some drugs such as Adriamycin, MTX, and vinblastine to penetrate through cell layers appears to be poor (11). Most alkylating agents are small molecules which tend to have good distribution into solid tumors. Finally, many agents, such as bleomycin, VCR, and etoposide, are more active against normally oxygenated cells (13, 14). The alkylating agents, in general, are equally toxic toward normally oxygenated and hypoxic cells (13).

While many tumors may have been clonal in origin, tumor progression has been related to the genetic instability of individual cells (15-17). Drug resistance, whether present in heterogeneous tumor cell populations at the outset or induced during the course of treatment, is a major therapeutic problem (3, 17, 18). Studies, using both cultured cell lines and tumor lines *in vivo*, indicate that resistance can be developed more quickly and to much greater levels to antimetabolite and antibiotic antitumor agents than to alkylating agents (19).

In this report, we define and discuss the effect of alkylating agent dose on the survival of the MCF-7 human breast carcinoma cell line and several MCF-7 alkylating agent-resistant sublines *in vitro* and the survival of FSAiC and EMT6 murine tumor cells and bone marrow CFU-GM treated *in vivo*. The discussion is framed in the context of the clinical therapeutic relevance of these results, and an attempt is made to relate findings from these preclinical studies to the known clinical pharmacology of these drugs.

MATERIALS AND METHODS

Drugs. HN2, BCNU, L-PAM, VCR, ara-C, and thiotepa were obtained from the Dana-Farber Cancer Institute pharmacy. HN2 as the hydrochloride salt was dissolved in 0.1 M HCl. In this form it remains stable for up to 1 year at -20°C (9). Aliquots were thawed and used immediately. BCNU-lyophilized powder was dissolved in 95% ethanol and stored, protected from light, at 4°C. This preparation results in 10% degradation in 78 days (20). L-PAM was dissolved in HCl-acidified ethanol and diluted in serum-free DME just before use. ara-C and VCR were diluted with DME just before use. CDDP pure powder was a gift from Johnson Matthey (Malvern, PA) and was dissolved in DME just prior to use. 4-HC was kindly provided in powder form by M. Colvin of Johns Hopkins University (Baltimore, MD) and was prepared in DME just prior to use. Mitomycin C was purchased from Sigma Chemical Co. (St. Louis, MO) and was dissolved in H₂O and diluted with DME just before use. MTX was a gift from the Pharmaceutical

² The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; CDDP, cisplatin [cis-diamminedichloroplatinum (II)]; HN2, nitrogen mustard; BCNU, *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea; L-PAM, melphalan, L-phenylalanine mustard; CPA, cyclophosphamide; 4-HC, 4-hydroperoxycyclophosphamide; MTX, methotrexate; thiotepa, trimethyleneiminethiophosphoramidate; VCR, vincristine; PBS, phosphate-buffered saline; DME, Dulbecco's minimal essential medium; IC₉₀, 90% inhibitory concentration; IC₅₀, 50% inhibitory concentration; CFU-GM, granulocyte-macrophage colony-forming unit; AUC, area under the curve.

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Resource Branch, National Cancer Institute (Bethesda, MD). MTX and thiotepa were dissolved in H₂O and diluted with DME just before use. For *in vivo* testing, drugs were prepared as above except that PBS was used as the final diluent.

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 (21, 22). MCF-7 human breast carcinoma cells grow as monolayers in DME supplemented with antibiotics, L-glutamine, and 10% fetal bovine serum. This cell line has a plating efficiency of 25–40%.

Dose Escalation. Nearly confluent 100-mm³ dishes of MCF-7 cells were treated for 1 h with approximately the concentration of each drug which would kill 90% of the cells, washed 3 times with 0.09% PBS, then covered with fresh medium plus serum. The concentration of alkylating agent was escalated at a rate of 15–20% per week and the cells were treated weekly unless there was no evidence of cell growth between treatments. The cells were “rested” (*i.e.*, not treated) only if there was danger of losing the line. Repeated attempts were made to escalate the drug treatment beyond the plateau concentrations. After 14 months of treatment, attempts were made to clone alkylating agent-resistant sublines from the treated cultures (19). Resistant sublines were screened for degree of resistance, generation times similar to those of the parent line, and relative stability of resistance (up to 2 months). Every 2 months, a vial of early-passage cloned cells was used to ensure that all experiments were carried out with the same subline. Generation times for the cell lines used in these studies were: MCF-7 parent line, 36 h; MCF-7/CDDP subline, 78 h; MCF-7/4-HC subline, 67 h; and MCF-7/L-PAM subline, 48 h.

Survival Curves. Parental MCF-7 cells or cloned alkylating agent-resistant sublines in exponential growth were treated with various doses of the drugs. After exposure to the agent or vehicle for 1 h in media without serum, for 24 h in media with dialyzed serum for MTX treatment, or for 24 h in media with fetal bovine serum for ara-C and VCR treatments, the cells were washed 3 times with 0.09% PBS solution and suspended by treatment with 0.25% trypsin/0.1% EDTA. The cells were plated in duplicate at 3 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 Lines. The FSaII fibrosarcoma (23, 24) adapted for growth in culture (FSaIIc) (24) was carried in male C3H/FeJ mice (The Jackson Laboratory, Bar Harbor, ME). For the experiments, 2 × 10⁶ tumor cells prepared from a brei of several stock tumors were implanted i.m. into the legs of 8- to 10-week-old male C3H/HeJ mice.

The EMT6 murine mammary carcinoma is an *in vivo-in vitro* tumor system (25–28). The EMT6 tumor was carried in BALB/c mice (Taconic Farms, Germantown, NY). For the experiments, 2 × 10⁶ tumor cells prepared from a brei of several stock tumors were implanted i.m. into the legs of BALB/c mice 8 to 10 weeks old.

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. When the tumors were approximately 50 mm³ in volume (about 1 week after tumor cell implantation), the drugs were administered as single doses by i.p. injection (0.2 ml). 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 suspension was prepared as described previously (29). The untreated tumor cell suspensions had a plating efficiency of 8–12%. The results are expressed as the surviving fraction ± SE of cells 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 (29). 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 expressed as the surviving fraction of treated groups compared to untreated controls.

Data Analysis. Quantitative analysis of survival curves was performed with the log-probit iterative least-squares method of Litchfield and Wilcoxon (30) as revised by Tallarida (31). Calculations were performed on an Apple II+ microcomputer.

RESULTS

In the study of drug dose effects, the development of drug-resistant cell lines has been a useful approach. The results of dose-escalation experiments with MCF-7 cells with three alkylating agents are shown in Fig. 1. The plateau in dose escalation with CDDP was reached after 6–8 months of treatment at a level 40 times the initial exposure concentration. Four to 6 months were required to escalate the dose of L-PAM to the maximum tolerated concentration, about 47 times the initial exposure concentration. This cell line reached the plateau in dose escalation of 4-HC in 6 months, at about 36 times the initial exposure concentration. Despite repeated attempts, it was not possible to maintain viable cultures at exposure concentrations above those achieved at these plateau levels.

Cloned cell lines were developed from these heterogeneous cultures approximately 14 months after the initiation of treatment. Only cloned lines which have cytokinetic properties similar to those of the parent cell line were selected for study. The survival of each of the three cloned resistant sublines and the parental MCF-7 line toward the agents to which resistance was developed are shown in Fig. 2. The MCF-7/CDDP line is about 6.5-fold resistant at the IC₅₀ concentration. There are over 2 logs of difference in cell kill between the parent MCF-7 line and the resistant subline at 250 μM CDDP and about 3 logs of difference in cell kill at 500 μM CDDP. The cloned MCF-7/L-PAM subline is about 7-fold resistant to the drug at the IC₅₀ concentration compared to the parental MCF-7 line. At a concentration of 50 μM L-PAM, there are nearly 3 logs of difference between the cell kill in the parental cell lines and the MCF-7/L-PAM-resistant subline. The cloned MCF-7/4-HC cell line is about 9-fold resistant to 4-HC at the level of 1 log of cell kill. At a 4-HC concentration of 250 μM there are greater than 2 logs of difference between the cell kill in the parental MCF-7 cell line and the MCF-7/4-HC-resistant subline.

The cross-resistance of these three cell lines to other alkylating agents was examined. Table 1 shows the resistance ratios at a level of 1 log of cell kill of each of these lines compared to

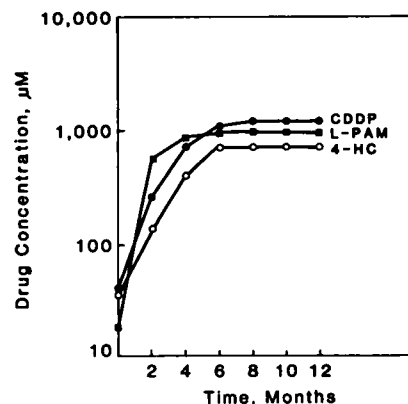


Fig. 1. Time course of drug concentration escalation for the MCF-7 human breast carcinoma cell line exposed to CDDP, L-PAM, or 4-HC.

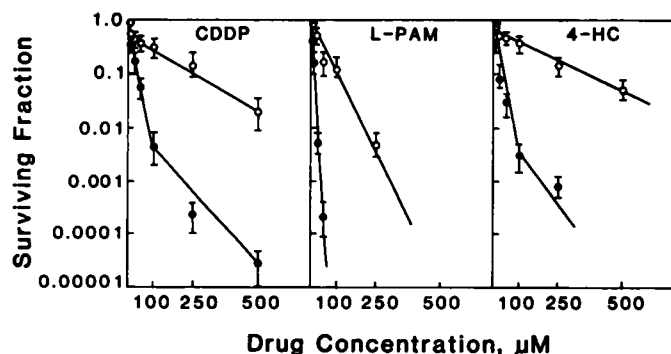


Fig. 2. Survival of the MCF-7 human breast carcinoma parent cell line (●) and cloned resistant MCF-7 sublines (○) to the drugs to which resistance was developed. Points, means of three independent experiments; bars, SE.

Table 1 Resistance ratios^a to various alkylating agents

Cell line	Drug						
	HN2	L-PAM	CDDP	BCNU	4-HC	thiotepa	Mito C
MCF-7/CDDP	1.6	2.0	6.5 ^b	1.4	1.1	3.1	1.3
MCF-7/L-PAM	5.6	7.0	4.9	0.2	5.0	3.5	3.0
MCF-7/4-HC	2.0	1.0	1.3	1.1	9.0	2.0	1.0

^a Resistance ratio = IC_{50} of resistant line/ IC_{50} of parental line. Drug exposure was for 1 h. The IC_{50} s (μ M) for the MCF-7 parental line were: HN2, 2.5; L-PAM, 15; CDDP, 40; BCNU, 355; 4-HC, 35; thiotepa, 140; and mitomycin C (Mito C), 2.

^b *Italicized numbers*, resistance ratio for the specific selected cell line.

the parental MCF-7 line for a variety of alkylating agents. A resistance ratio of 3 or greater was taken to indicate a significant level of resistance of a subline to a given agent as compared to the parental cell line. The MCF-7/CDDP line showed no cross-resistance to the other drugs except for a small effect seen with thiotepa. The MCF-7/L-PAM cell line showed a very interesting pattern of cross-resistance. This cell line, which was about 7-fold resistant to L-PAM, was also about 5-fold resistant to HN2, CDDP, and 4-HC. It was also about 3-fold resistant to thiotepa and mitomycin C. However, this cell line was 5-fold more sensitive to BCNU than was the parental cell line. The MCF-7/4-HC cell line was quite selectively resistant to 4-HC and showed no significant cross-resistance to other alkylating agents.

Survival curves for the MCF-7/L-PAM cell line and the parental MCF-7 line toward CDDP, BCNU, 4-HC, and HN2 are shown in Fig. 3. As can be seen, the relatively small differences in cell kill seen as resistance ratios do not necessarily predict the differences seen at higher levels of cell kill. At the higher concentrations of CDDP there are greater than 2 logs of difference between the cell kill seen in the parental MCF-7 cell line and the L-PAM-resistant subline. With BCNU at 250 μ M there were greater than 5 logs of cell kill in the MCF-7/L-PAM cell line and less than 1 log of kill in the parental MCF-7 cell line. The survival of the MCF-7/L-PAM cell line exposed to 4-HC may reflect the survival of a resistant subpopulation within the parental cell line population. At a concentration of 250 μ M 4-HC, there are about 2 logs greater cell kill in the MCF-7 parental cell line than in the MCF-7/L-PAM line. The MCF-7 parental and MCF-7/L-PAM cell survival curves have different slopes in response to exposure to HN2. There was about a 2-log difference in cell kill between the two lines at a concentration of 10 μ M HN2. In Fig. 4, survival data for the MCF-7 parental cell line toward both alkylating agents and other antineoplastic agents are presented as multiples of the IC_{50} concentrations for each treatment. Although alkylating agents continue to kill cells in a relatively log-linear manner

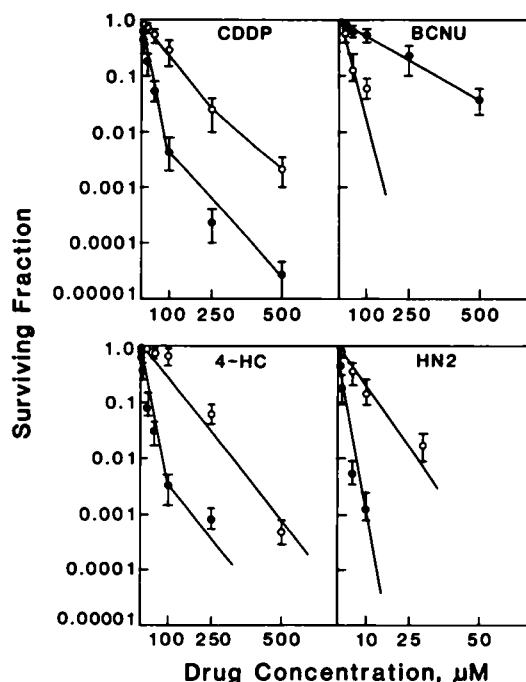


Fig. 3. Survival of the MCF-7 human breast carcinoma parent cell line (●) and of the MCF-7/L-PAM subline (○) exposed to various alkylating agents for 1 h. Points, means of three independent experiments; bars, SE.

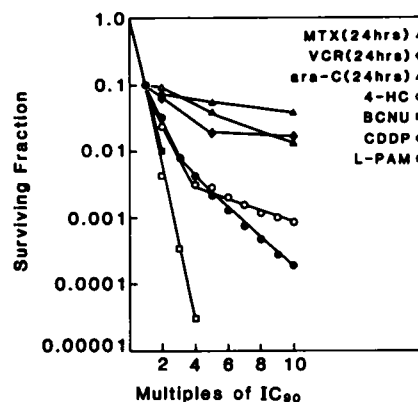


Fig. 4. Survival of the MCF-7 human breast carcinoma parent cell line exposed to several alkylating agents (4-HC, BCNU, CDDP, and L-PAM) for 1 h, or nonalkylating agents (MTX, VCR, and ara-C) for 24 h. The results are expressed as multiples of the IC_{50} for each drug.

with increasing concentration of the drugs, with other drugs (MTX, ara-C, and VCR) which are more cell cycle dependent, there is clearly a leveling off in cell kill despite increasing drug concentrations. It is interesting that the alkylating agent-resistant MCF-7 sublines also show log-linear increase in cell kill with multiples of the IC_{50} , and that the slopes of these survival lines are very similar to that of the parent MCF-7 line for the same drug (data not shown).

Using the FSaII murine fibrosarcoma and the EMT6 murine mammary carcinoma *in vivo-in vitro* tumor systems, the response of tumors *in vivo* was examined to increasing single doses of alkylating agents and MTX (Fig. 5). Tumor cell kill was quantified by colony formation and the survival of bone marrow from the same animals was measured by the CFU-GM assay. Five alkylating agents were used. As was found in culture, there was log-linear increase in tumor cell kill with increasing dose of the alkylating agents. For MTX there was a marked leveling off of the kill curve, whether the drug was given as a single injection or as three injections over 12 h, which is also

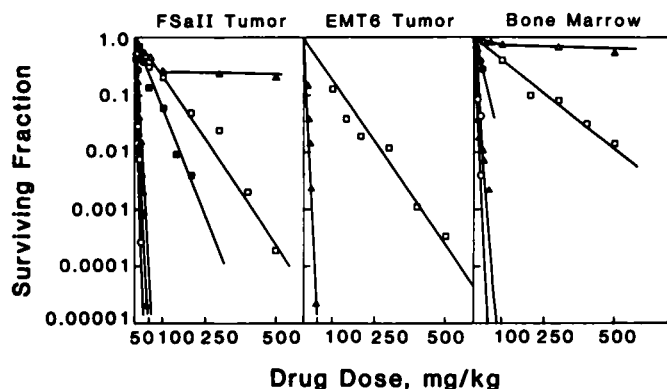


Fig. 5. Survival of FSaII tumor cells, EMT6 tumor cells, and bone marrow (CFU-GM) cells treated *in vivo* with single doses of CDDP (●), L-PAM (○), BCNU (■), CPA (□), thiotepa (Δ), or MTX (△). Points, mean of three independent determinations.

Table 2 Comparative *in vitro-in vivo* antitumor effects of alkylating agents

Drug	<i>In vitro</i> ^a concentration (μM) for cell kill of		<i>In vivo</i> ^b dose (mg/kg) for cell kill of		Ratio of <i>in vitro</i> concentration/ <i>in vivo</i> dose for cell kill at	
	1 log	3 logs	1 log	3 logs	1 log	3 logs
4-HC/CPA ^c	35	100	100	375	0.35	0.27
L-PAM	10	30	8	22	1.25	1.36
BCNU	300	>500	90	250	3.3	>2.00
Thiotepa	125	475	10	25	12.5	19
CDDP	22	170	10	20	2.2	5.0

^a Studies were performed in the human breast cancer MCF-7 cell line. Cell survival was assessed after 1 h of drug exposure.

^b Studies with CPA and thiotepa were performed with the EMT6 murine breast cancer tumor system; studies with L-PAM, BCNU, and CDDP were performed with the FSaII murine fibrosarcoma tumor system. Single doses of each drug were administered *i.p.*, 24 h prior to tumor excision.

^c 4-HC was used for *in vitro* studies.

consistent with the above *in vitro* studies. There was a similar log-linear correlation with respect to normal marrow CFU-GM; however, the slopes of the survival curves were more shallow than for the survival curves of the tumor cells.

The comparative *in vitro-in vivo* cytotoxicity of these drugs in terms of concentrations or dose of drug required to produce a 1 or 3 log kill of tumor cells was determined (Table 2). The *in vitro-in vivo* ratios for cell kill were calculated at both levels of drug action. There is relatively little evidence for *in vitro* biotransformation of L-PAM and, as is shown, the ratio of *in vitro* to *in vivo* activity of this drug is very close to 1 at both levels of cell kill. Ratios of 0.35 and 0.27 for 4-HC/CPA suggest that a significant proportion of the drug is inactivated or not activated *in vivo*. BCNU and CDDP have ratios which are relatively close to 1, indicating comparable activity in tissue culture and *in vivo*. On the other hand, ratios for thiotepa are 12.5 and 19, suggesting that thiotepa is transformed *in vivo* to a substantially more potent drug, an observation consistent with other evidence that thiotepa is converted to more active forms (32). This suggests that *in vitro* to *in vivo* interpretations for thiotepa activity should be made with caution. Table 3 presents several clinical pharmacological parameters for five alkylating agents. Table 3, Column 2 shows the approximate clinically accepted maximum safe single dose for each drug in mg/m² (33). The bioavailability derived from the AUC of each drug at that dose is shown in Column 3 (34–36). Finally, the ratio of bioavailability to drug dose is shown in the last column. The ratio of bioavailability to dose for thiotepa is comparable to that for L-PAM and CPA, indicating that the superiority of thiotepa *in vivo* presented in Table 2 is most likely not due to

Table 3 Clinical pharmacology of alkylating agents

Drug	Dose ^a (mg/m ²)	Measured bioavailability ^b AUC (μg·h/ml)	Bioavailability/dose ratio
4-HC/CPA	1000	160	0.16
L-PAM	20	2.5	0.12
BCNU	200	2.1	0.01
Thiotepa	60	4.5	0.08
CDDP	100	2.0	0.02

^a Approximate clinically accepted maximum safe single dose.

^b Bioavailability for the parent drug molecule (not metabolites) was obtained from Alberts and Chen (34), Struck *et al.* (35), and Alberts *et al.* (36). AUC is the area under the curve for plasma levels of the parent drugs over a time course after administration of a single dose of drug.

Table 4 Correlation of *in vitro* and *in vivo* findings

Drug	<i>In vitro</i> concentration, AUC		<i>In vivo</i> ^b dose, AUC		<i>In vitro</i> AUC/ <i>in vivo</i> AUC ratio
	μM ^a	μg·h/ml ^c	mg/m ²	μg·h/ml	
4-HC/CPA	35	5.6	1000	160	0.4
L-PAM	10	1.7	20	2.5	0.7
BCNU	300	48	200	2.1	22.9
Thiotepa	125	23.8	60	4.5	5.3
CDDP	22	6.6	100	2.0	3.3

^a Drug concentration which produces 1 log of cell kill from the survival curves of MCF-7 human breast carcinoma cells exposed to each drug for 1 h.

^b Data from references as shown for Table 3.

^c The calculation for drug exposure in μg·h/ml was made assuming that the half-life in solution of BCNU, 4-HC, and L-PAM was 30 min and that thiotepa and CDDP concentrations in solution are stable for 1 h.

thiotepa *per se*, but presumably due to its metabolites. The much lower ratio for BCNU (0.01) is consistent with the known extensive metabolism of this compound which, based on the ratio in Table 2, occurs to a substantial degree both *in vitro* and *in vivo*.

In Table 4 we have attempted an *in vitro-in vivo* correlation between data obtained with the MCF-7 human breast carcinoma cell line and the clinical pharmacological data for each of these drugs. We selected the MCF-7 line as a representative *in vitro* human tumor cell culture system because it is a well-characterized, relatively chemotherapy-responsive cell line (19). Table 4, column 2 shows the *in vitro* concentrations of the various alkylating agents required to produce 1 log of cell kill; in Column 3 this is converted to micrograms of parent drug present in the media over the 1 h of drug exposure time. This calculation was made by assuming that BCNU, 4-HC, and L-PAM had half-lives in solution of 30 min and that thiotepa and CDDP had stable concentration levels for 1 h. In Table 4, Column 3, the clinically maximum safe single dose that can be administered and the plasma levels (AUC) that these doses produced in circulation is presented. In Column 4, the *in vitro/in vivo* ratios of the AUCs are presented (33–36). If 1 log of kill is achieved *in vivo* against tumor stem cells with a maximum safe single dose of each drug and if the AUC produced with each drug *in vivo* is representative of the action of that drug *in vitro*, then the *in vitro/in vivo* ratio of the AUC should approach 1. These assumptions are most true in the case of L-PAM which is a simple nitrogen mustard molecule requiring no metabolic activation. CPA appears to be far more active *in vitro* (30-fold). This is because *in vitro* the activated form, 4-HC, is used which may also have transport advantages over CPA (37). On the other hand, *in vivo* CPA is extensively metabolized, including metabolism to inactive compounds (37). Thus, whereas for L-PAM direct *in vitro/in vivo* correlations might be reasonable and *in vitro* studies might be predictive, the situation for CPA is less secure. For BCNU a much higher concentration is required *in vitro* as compared to *in vivo* (22-fold) to produce the

same, or presumably the same, biological effects. This is also somewhat true for thiotepa. Both BCNU and thiotepa (38) are extensively metabolized and some of the metabolic products *in vivo* have antitumor activity which is greater than that of the parent compounds. CDDP is mainly activated intracellularly and this drug has comparable activity *in vitro* and *in vivo*.

DISCUSSION

The production of drug-resistant cell lines through increasing selection pressure has been a very successful means of producing resistance to antitumor antimetabolites such as MTX (39, 40) and antitumor antibiotics such as Adriamycin (41, 42). This methodology has been much more difficult to apply to the alkylating agents because of their generally short half-times in solution; however, a number of alkylating agent-resistant cell lines have been produced (8, 10, 19).

While it was possible to escalate the exposure dose to alkylating agents of cells *in vitro* 30- to 40-fold over the course of 3-6 months, increasing selection pressure further was not possible and resulted in cell death. In many cases resistance was unstable upon removal of selection pressure, perhaps due to back mutation (43). Thus, there appears to be a limitation to the degree of resistance which can be produced to alkylating agents, even under optimal conditions of selection pressure. The recent clinical trials of combined high-dose alkylating agent treatment with autologous bone marrow transplantation take advantage of the difficulty in surpassing even 10-fold resistance to alkylating agents (2-7). This resistance "ceiling" at relatively low drug levels contrasts with other agents, particularly MTX, where, with continued selection pressure in culture, very high levels (10^4 -fold increase) of resistance can be achieved (3, 40, 44), and with natural products such as Adriamycin where levels of resistance achieved often closely match the level of selection pressure (41, 42, 45). In contrast, the greatest level of resistance which can be achieved to ionizing radiation is through the application of hypoxia and then a maximum of 3-fold resistance is possible. The plateau of dose escalation for alkylating agents may reflect an inability of the cells to activate a gene amplification mechanism through which greater drug resistance could be achieved. This low ceiling for alkylating agent resistance has very important clinical implications and provides a strong rationale for high-dose alkylating agent clinical regimens. The multifactorial nature of drug resistance and the lack of cross-resistance among alkylating agents has major implications for both dose and combination alkylating agent strategies in the clinic.

We have previously reported non-cross-resistance among alkylating agents to be true for a variety of human cell lines and find in the present study that as a generality there is no cross-resistance with these MCF-7 sublines (Table 1). This is clearly the case for the MCF-7 lines resistant to CDDP and 4-HC. On the other hand, for the line resistant to L-PAM there is partial cross-resistance to HN2, CDDP, and 4-HC. Interestingly, this line exhibits 5-fold collateral sensitivity to BCNU. These patterns of cross-resistance or lack thereof have major implications with respect to mechanisms of resistance. They also have major implications for the dose effect of alkylating agents in combination and for the development of combination alkylating agent regimens. Thus, with two non-cross-resistant, independently acting agents wherein 10^{-4} cells in the tumor population were resistant to each agent, a maximum of 10^{-8} tumor cells could be destroyed by the combination. For two similarly effective alkylating agents which exhibited maximum cross-resistance,

cell kill for the combination might still be in the range of 10^{-4} tumor cells.

The resistance expressed in Table 1 is the ratio of the IC_{50} s for the resistant line to the parent line. It is very important, however, to look at resistance at higher drug concentrations and over many logs of tumor cell kill. When this is done (Fig. 2), it can be seen that with the alkylating agents the log-linear relationship between drug concentration and cell kill is maintained, but that the survival curves for the resistant sublines *versus* those of the parent cell line are displaced by approximately a constant multiple. For example, a 5-fold increase in L-PAM concentration in the MCF-7/L-PAM cell line produces the same log kill in the resistant cells as does a unit dose increase of L-PAM in the MCF-7 parent cell line. Thus, the resistance or sensitivity of these cells to alkylating agents is relative and the pattern of the dose-response relationship of the cells to drugs remains the same. Looking at the data in Fig. 2 in another way, at high concentrations (presumably transplant concentrations), there is a 3- to 4-log advantage against the parent cell line as compared to the resistant subline for each drug.

Cross-resistance is commonly measured at a fixed biological end point, such as IC_{50} or IC_{90} . With increasing concentrations of drug, tumor cell kill may be a log-linear function of dose, but there is often a deviation of the dose-response curve at very high levels of cell kill. Moreover, IC_{50} s may obscure the fact that at substantially higher drug concentrations alkylating agents may continue to kill cells in a log-linear manner, whereas with many nonalkylating agents there is no continued increase in tumor cell kill with increasing dose of drug.

A major advantage of the alkylating agents over most non-alkylating agents is demonstrated in Fig. 4, where the effect on the MCF-7 breast cancer cell line is expressed as multiples of the IC_{90} . With the alkylating agents, linearity is reasonably well maintained down through 4 to 5 logs of cell kill. On the other hand, with the nonalkylating agents, linear increase in cell kill with dose is lost at approximately 2 logs. These studies were conducted at several drug exposure times to reduce cytokinetic factors as a mechanism for leveling of the dose effect. Thus, it is proposed that a major contributor to deviation from linearity in the multilog killing curves is the heterogeneity in sensitivity to each drug of a given tumor cell population. This would indicate that the multilog killing curve method of study and analysis has clinical relevance.

The dose-response studies of alkylating agents discussed above were conducted with cell lines growing as monolayers *in vitro*. Many cancer chemotherapeutic agents, particularly some of the alkylating agents, are extensively biotransformed by the host. One should therefore interpret *in vitro* studies with caution and perform parallel *in vivo* studies wherever possible. Accordingly, we performed such studies by using the *in vivo-in vitro* tumor excision assay in two murine solid tumor models (Fig. 5). There continued to be a linear increase in tumor cell killing with levels of alkylating agents well above lethal doses. These data provide a very strong rationale for the use of alkylating agents both singly and in combination in a clinical bone marrow transplant setting. On the other hand, for MTX there was less than 1 log of kill and a leveling off of the dose-response curve, using both single and multiple dose schedules.

Because there were several *in vitro/in vivo* differences in drug behavior and because of the importance of being able to extrapolate these laboratory findings to clinical situations, we attempted a more detailed analysis of the preclinical data in association with known clinical drug levels (Tables 2-4). In

Table 2, the *in vitro* concentrations required to produce a 1 or 3 log tumor cell kill of MCF-7 parent cells was compared with the *in vivo* dose required to produce a 1 or 3 log kill of tumor cells from the tumor excision assay. The assumptions involved are apparent, but the important data in Table 2 are the comparative *in vitro/in vivo* ratios for the different alkylating agents. This ratio is nearest to 1 for L-PAM. It can be seen that CPA is considerably less active *in vivo* than is 4-HC *in vitro*, whereas thiotepa is comparatively far more active *in vivo* than *in vitro*. This is consistent with the observation that CPA is extensively metabolized to both active and particularly inactive metabolites (36), whereas thiotepa is known to be activated by hepatic microsomal enzymes (31).

In Table 3, the approximate maximum safe single dose in patients of the individual alkylating agents is related to the bioavailability of the parent compound for that dose (33-35), and the ratio of bioavailability to dose is rendered. The relatively low ratios for BCNU is due to its extensive metabolism and for CDDP to its extensive protein binding.

In Table 4, the AUC estimated to produce 1 log of cell kill in MCF-7 cells *in vitro* was related to the AUC achieved by the maximum safe single dose of the alkylating agents in patients. Again, for CPA the ratio was very low, consistent with the fact that most of the metabolic products are inactive (36). For BCNU and thiotepa, the opposite was true.

The calculations presented in Tables 2-4 and the data in the figures clearly show quantitative discrepancies for cytotoxicity for the alkylating agents *in vivo* compared to *in vitro*. While there are many assumptions involved in these correlations, the data clearly support the importance of *in vivo* assay of drug action. The tumor excision assay has two important qualities: (a) pharmacological biotransformation has occurred, and (b) a quantitative multilog cell kill assay is possible *in vivo*. Such information can suggest important pharmacological properties of the chemotherapeutic agents, and major *in vitro-in vivo* differences suggest caution in extrapolating *in vitro* data to the clinic. The strength of this assay is that it is quantitative; however, qualitative differences are also possible. For example, resistance developed by *in vivo* passage of tumor cells may not lead to the same mechanisms of resistance as *in vitro* passage of tumor cells. On the other hand, resistance developed by *in vitro* passage may not be as relevant to the clinical problem if such resistance does not apply to the active *in vivo* biotransformation products of these drugs. We are currently developing alkylating agent-resistant sublines of the EMT6 murine mammary carcinoma *in vitro-in vivo* tumor line.

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