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Antitumor alkylating agents: in vitro cross-resistance and collateral sensitivity studies

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Abstract. Cell lines resistant to five antitumor alkylating agents (CDDP, PAM, 4-HC, HN2, and BCNU) were developed from five parental human tumor lines representative of solid tumors with a range of sensitivities to antitumor alkylating agents. The parental cell lines were SCC-25 squamous carcinoma of the head and neck, MCF-7 breast carcinoma, SW2 small-cell lung cancer, SL6 non-small-cell lung carcinoma, and G3361 melanoma. Survival curves using colony formation as the endpoint were generated for each of the 25 cell lines to each of the five alkylating agents. Comparison of the drug concentrations that reduced the survival of the alkylating agent-resistant cell lines by 90% (IC_{90} values) with the IC_{90} values obtained for the corresponding parental cell lines was used as a measure of the resistance/sensitivity of the alkylating agent-resistant lines to each drug tested. Although cross-resistance among the alkylating agents was generally uncommon, several patterns of response emerged. Cross-resistance occurred in 27 of the 105 determinations and occurred most frequently in the cell lines in which resistance was developed to PAM (57%) or BCNU (38%). Cross-resistance to HN2 occurred most frequently. Collateral sensitivity was equally as common, occurring in 25 of the 105 determinations. Collateral sensitivity occurred most frequently in the cell lines made resistant to 4-HC. The 4-HC-resistant cell lines were most frequently collaterally sensitive to PAM and to BCNU. Cross-resistance developed most frequently in the MCF-7 breast carcinoma and SCC-25 squamous-cell carcinoma cell lines, whereas collateral sensitivity developed most frequently in the SW2 small-cell lung cancer line and the G3361 melanoma cell line and least frequently in the MCF-7 breast carcinoma cell line and the SL6 non-small-cell lung cancer cell line. The implication of these findings for the development of strategies for clinical treatment are discussed.

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Abbreviations: AAs, antitumor alkylating agents; CDDP, cisplatin, *cis*-diamminedichloroplatinum(II); HN2, nitrogen mustard; BCNU, *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea; PAM, L-phenylalanine mustard, melphalan; 4-HC, 4-hydroxyperoxycyclophosphamide; CPA, cyclophosphamide; THIO, trimethyleneiminethiophosphoramide; DME, Dulbecco's modified Eagle's medium; IC_{90}, 90% inhibitory concentration; PBS, phosphate-buffered 0.9% saline; FBS, fetal bovine serum; GSH, glutathione; GST, glutathione-S-transferase

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Introduction

Antitumor alkylating agents (AAs) have been considered to be a relatively homogeneous class of compounds. AAs were described as radiomimetic agents and were considered to be cross-resistant with each other, primarily on the basis of clinical impressions [43]. These generalizations were challenged by the classic data of Schabel et al. [35–37], which indicated that in tumor sublines of the L1210 murine leukemia system made resistant to specific AAs, cross-resistance among the AAs was the exception rather than the rule [39], and, more recently, by data accrued in several human tumor lines [17, 19, 20, 43–47, 51]. We have confirmed the findings of Schabel et al. in our studies in human tumor cell lines, which indicated that cross-resistance among, the AAs is indeed the exception rather than the rule [17, 19, 20, 37, 44–47, 51]. Chemically, AAs are quite heterogeneous, leading to different mechanisms of resistance to specific agents within the class. Similarly, other determinants of drug action, such as plasma membrane transport and intracellular biotransformation (activation or inactivation), vary substantially among AAs [21, 22, 32, 49]. The mechanisms of resistance to AAs are multiple and multifactorial, consistent with the general lack or, at best, low levels of cross-resistance among these drugs [17, 19, 20, 43, 45–47, 51].

There has been renewed interest in the AAs in recent years largely because, as a class, they are ideal agents for

high-dose marrow-protection treatment protocols since their dose-limiting toxicity is often myelosuppression [9, 14, 16, 30, 31, 48]. Most major advances in clinical chemotherapy have involved combinations of chemotherapeutic agents [3, 13]. The knowledge of the mechanisms of action, the know dose-limiting toxicities, and the observations concerning lack of cross-resistance provide the rationale for the use of AAs in combination in high-dose marrow-protection studies in the clinic [3, 13, 16, 18, 24, 39].

In addition to the aforementioned generalizations concerning resistance and cross-resistance, we have observed interesting and potentially therapeutically relevant examples of collateral sensitivity among the AAs. An analysis of the biology of resistance, cross-resistance, and collateral sensitivity of the AAs is the central theme of this report. The biochemical basis for this biology is under investigation.

Materials and methods

Drugs

HN2 and BCNU were obtained from the Dana-Farber Cancer Institute pharmacy. HN2 as the hydrochloride salt was resuspended in 0.1 *M* HCl. In this form it remains stable for up to 1 year at –20° C [21]. Aliquots were thawed and used immediately. BCNU lyophilized powder was resuspended in 95% ethanol and stored under protection from light at 4° C. This preparation results in 10% degradation in 78 days [7]. PAM and THIO were purchased from Sigma Chemical Co. (St. Louis, Mo.). PAM was dissolved in HCl-acidified ethanol and diluted in serum-free DME just prior to use. 4-HC was kindly provided as a gift by Dr. J. Pohl (Asta Medica, Frankfurt am Main, Germany). 4-HC was prepared in DME just before use. CDDP pure powder was a gift from Johnson-Matthey, Inc. (Malvern, Pa.).

Cell lines

SW2 small-cell lung carcinoma. The SW2 cell line was initiated from pleural fluid obtained from a patient with small-cell carcinoma [11, 12]. These cells grow exponentially in RPMI 1640 (Gibco, Grand Island, N. Y.) supplemented with 10% FBS (Sterile Systems, Logan, Utah) and antibiotics as enlarging spheroids with a doubling time of 2–4 days, eventually reaching a plateau by day 30. The spheroids were dispersed to make a single-cell suspension for drug exposure. Colonies were grown in soft agar, and the plating efficiency of this cell line is 10%–15%.

SL6 lung adenocarcinoma. The SL6 cell line was developed from a lung mass obtained from a man with large-cell carcinoma. This cell line grows as a monolayer in RPMI 1640 medium supplemented with 10% FBS and antibiotics and has a plating efficiency of 45%–60% [44].

MCF-7 breast carcinoma. The MCF-7 human adenocarcinoma of the breast cell line was 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 human breast carcinoma cells grow as monolayers in DME supplemented with antibiotics, L-glutamine, and 10% FBS [25, 42]. This cell line has a plating efficiency of 25%–40%.

SCC-25 squamous-cell carcinoma. The SCC-25 cell line (human squamous carcinoma of the head and neck) retains an epithelioid appearance and grows without the aid of a feeder layer [34]. It has a plating efficiency of 10%–35%. The cells grow in DME supplemented with 10% FBS, antibiotics, and hydrocortisone (0.4 μg/ml) [17].

G3361 melanoma. The G3661 cell line was derived from a single cell that had been obtained from a biopsy of human melanoma and had been cloned in soft agar. This cell line has an 85%–95% plating efficiency and grows as a monolayer. It is heavily pigmented and has a human polyploid karyotype. A high level of tyrosinase activity in the melanin and melanin-grain microstructure indicates that this cell line retains its differentiated phenotype [54]. The G3361 cell line was grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics.

Dose escalation

The parent cells were treated for 1 h with the concentration of each drug that would kill 90% of the cells, washed three times with PBS, then covered with fresh medium plus serum. The concentration of AA was escalated at a rate of 15%–20%/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 culture. Repeated attempts were made to escalate the drug treatment beyond the plateau concentrations. After 14 months of treatment, sublines were cloned from the treated cultures [44]. Resistant sublines were screened for degree of resistance, similarity of generation time to that of the parent line [33], and relative stability of resistance (at 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.

Survival curves

Cells in exponential growth were treated with various concentrations of the drugs. After exposure to the agents or vehicle for 1 h in medium without serum, the cells were washed three times with PBS and the monolayer was suspended by treatment with 0.25% trypsin/0.1% ethylenediaminetetraacetic acid (EDTA). 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 more were counted. The small-cell lung cancer cells were grown in suspension and were plated in 0.5% soft agar as described above. Results were expressed as the surviving fraction of treated cells as compared with vehicle-treated control cells [43, 46, 47].

Data analysis and definitions

The natural log of cell kill was regressed against the AA concentration for each cell-survival curve through the origin with an extra (interaction) term, the product of concentration and a 0–1 dummy variable indicating the cell-survival linear curve. The slopes of these (linear) regression lines were then compared in a standard analysis of variance by testing whether the parameter estimate of the extra term was zero [8]. In the event that the estimate was significantly different from zero, we concluded that the two slopes differed significantly. Several of these analyses were performed. For more ready manipulation of data in tabular form, IC_{90} values were used as described below.

The drug concentration that reduced the survival of the cells by 90% (surviving fraction, 0.1) was designated the IC_{90} value. This value was selected as a point of comparison because (1) the IC_{90} is equivalent to a good partial or complete clinical response and is thus a recognizable and quantifiable endpoint in the clinic, (2) the IC_{90} is always on the straight-line portion of the log/linear cell-survival curves for AAs and is therefore a good representation of the survival curves, and (3) analysis of whole survival curves or slopes as described above gave results and ratios similar to those obtained using comparisons of IC_{90} values. The resistance ratio is equal to the IC_{90} value obtained for the resistant cell line divided by that found for the parent cell line; for example, the IC_{90} of SCC-25/PAM (selecting agent) to PAM (challenging agent) divided by the IC_{90} of SCC-25/parent to PAM was 5.5 (see Table 2).

The cross-resistance (or collateral sensitivity) ratios were designated by degree:

1. Major cross-resistance indicates that the resistance ratio of the challenging agent is greater than two-thirds that of the selecting agent; for

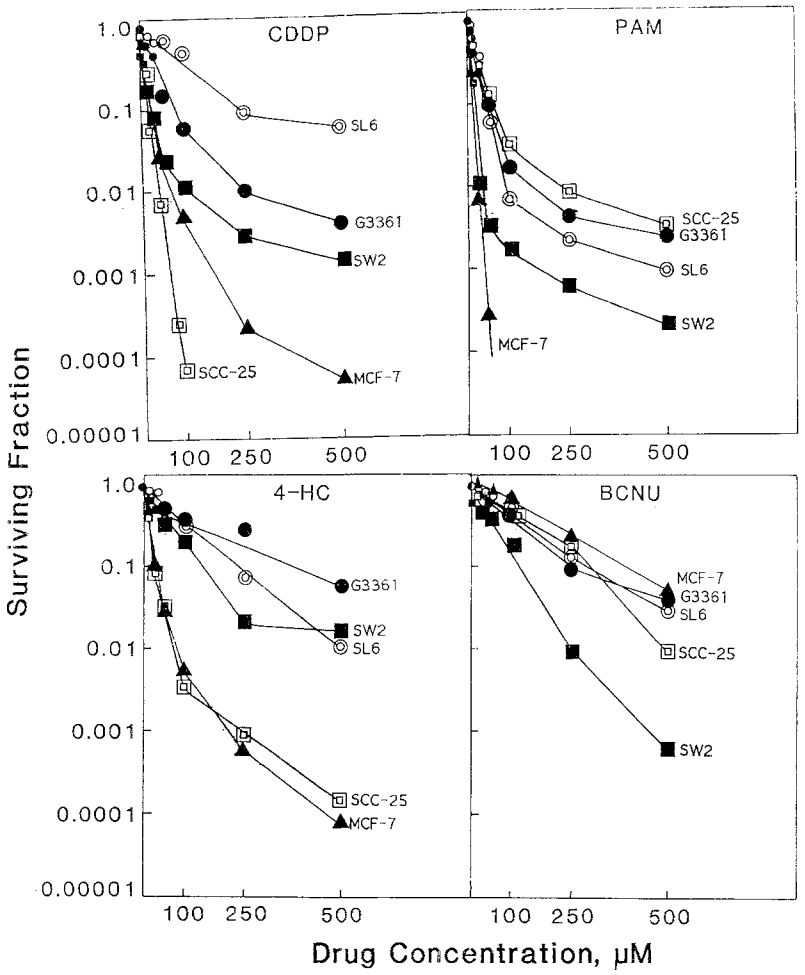

Fig. 1. Survival of G3361 melanoma cells (●), SL6 lung adenocarcinoma cells (○), SW2 small-cell carcinoma cells (■), SCC-25 head and neck carcinoma cells (□), and MCF-7 breast carcinoma cells (▲) exposed to various AAs for 1 h. The results are presented as the mean values for three independent experiments

example, the resistance ratio of SCC-25/PAM to HN2 (challenging agent) is 6.3, whereas the resistance ratio of SCC-25/PAM to PAM (the selecting agent) is 5.5, i.e., greater than two-thirds that of the selecting agent (see Table 2).

2. Moderate cross-resistance is the same except that the ratio limits are less than two-thirds the resistance ratio of the selecting agent, but the minimal value is 2.3.

3. No cross-resistance is indicated by resistance ratios ranging between 0.7 and 2.2; resistance ratios inclusive of these limits were found to be statistically not different than values of 1.0.

4. Major collateral sensitivity is defined as resistance ratios less than or equal to 0.3.

5. Moderate collateral sensitivity is defined as resistance ratios ranging between 0.3 and 0.7.

Results

The five parental human tumor cell lines chosen for this study included (1) a squamous-cell carcinoma of the head and neck (SCC-25), (2) a breast carcinoma (MCF-7), (3) a small-cell lung cancer (SW2), (4) a non-small-cell lung adenocarcinoma (SL6), and (5) a melanoma (G3661). These cell lines were chosen because each exhibits features relevant to its lineage. Thus, the breast cancer cell line is positive for estrogen receptors and the melanoma cell line produces melanin [25, 42, 54].

The relevance of these cell lines to the clinically observed responsiveness of the various tumor types to antitumor AAs is important to the interpretation and conclusions drawn in this study. The survival of the five cell lines after exposure to various concentrations of the antitumor AAs for 1 h is shown in Fig. 1. The surviving fraction is presented in a log scale on the Y axis as a function of linear increase in AA concentration on the X axis. The level of cytotoxicity of the AAs can be evaluated (1) by the slope of the linear portion of the survival curve and (2) as a function of the level and degree of curvilinearity of the survival curve. These measures are approximated by comparisons of IC_{90} values in the following analyses. By these criteria, the melanoma G3361 cell line is the least sensitive to the AAs overall and the non-small-cell lung-cancer SL6 cell line is moderately sensitive, whereas the MCF-7 breast carcinoma cell line, the SW2 small-cell lung-cancer cell line and the SCC-25 squamous carcinoma cell line are relatively sensitive [44]. These trends correspond reasonably well to the known clinical activity of the various antitumor AAs against the corresponding neoplasms. A semiquantitative ranking of the activity of the various AAs in the five cell lines from 0 (no activity) up through 4+ (representing steep survival curves) is presented in Table 1. These are compared with a semiquantitative ranking of the clinical antitumor activity of the AAs in the disease of origin of each cell line. A reasonably good correlation is obtained [20]. These comparisons suggest that our in vitro models correlate with

Table 1. Activity of the various AAs in clinical cancer treatment and in human tumor cell lines derived from the same tumor types[a]

Tumor type	AAs							
	CDDP		CPA		PAM		BCNU	
	Clinic	Culture	Clinic	Culture	Clinic	Culture	Clinic	Culture
Squamous carcinoma of the head and neck	3+	4+	1+	1+	2+	3+	1+	1+
Breast carcinoma	3+	3+	4+	4+	3+	3+	2+	1+
Small-cell lung cancer	3+	4+	2+	3+	2+	3+	2+	2+
Non-small-cell lung cancer	2+	0	0–1+	1+	1+	1+	1+	1+
Melanoma	0	1+	0–1+	0	0	1+	1+	1+

[a] Semiquantitative ranking, where 0 = no activity and 4+ = maximal activity (see Results)

Table 2. Resistance ratios of the various AA-resistant human tumor cell lines to various AAs[a]

Cell lines	AAs					
	CDDP	PAM	4-HC	HN2	BCNU	THIO
Squamous carcinoma:						
SCC-25	1.0	1.0	1.0	1.0	1.0	1.0
SCC-25/CDDP	**30.0**	5.0	3.0	1.8	2.0	1.8
SCC-25/PAM	2.7	**5.5**	3.6	6.3	1.1	–
SCC-25/4-HC	0.5	0.1	**4.5**	6.7	0.2	–
SCC-25/HN2	1.3	0.3	1.2	**6.7**	0.6	1.7
SCC-25/BCNU	1.4	0.1	2.4	4.3	**3.5**	–
Breast carcinoma:						
MCF-7	1.0	1.0	1.0	1.0	1.0	1.0
MCF-7/CDDP	**6.5**	2.0	1.1	1.6	1.4	3.1
MCF-7/PAM	4.9	**7.0**	5.0	5.6	0.2	3.5
MCF-7/4-HC	1.3	1.0	**9.0**	2.0	1.1	2.0
MCF/HN2	2.3	0.8	1.3	**5.5**	0.7	1.7
MCF-7/BCNU	4.0	4.3	1.3	20.0	**2.7**	1.9
MCF-THIO	–	–	–	–	–	**7.0**
Small-cell lung cancer:						
SW2	1.0	1.0	1.0	1.0	1.0	1.0
SW2/CDDP	**3.3**	0.7	0.2	1.0	1.8	–
SW2/PAM	6.7	**3.3**	0.3	2.5	0.3	–
SW2/4-HC	2.0	0.1	**4.7**	1.0	0.5	–
SW2/HN2	3.0	0.7	0.3	**4.0**	0.9	–
SW2/BCNU	1.0	0.5	0.3	3.0	**4.2**	–
Non-small-cell lung cancer:						
SL6	1.0	1.0	1.0	1.0	1.0	1.0
SL6/CDDP	**3.5**	1.3	1.5	1.5	0.9	1.1
SL6/PAM	0.8	**4.0**	2.3	4.4	0.9	–
SL6/4-HC	1.3	0.1	**5.0**	1.0	0.9	–
SL6/HN2	2.3	0.8	1.2	**2.5**	0.7	–
SL6/BCNU	1.2	1.5	1.1	1.7	**4.6**	–
Melanoma:						
G3361	1.0	1.0	1.0	1.0	1.0	1.0
G3661/CDDP	**9.2**	2.0	1.5	1.7	0.6	1.2
G3661/PAM	0.4	**4.0**	0.1	5.0	0.8	–
G3661/4-HC	0.5	0.5	**5.4**	0.3	0.4	–
G3661/HN2	–	–	–	**6.1**	–	–
G3661/BCNU	0.3	5.5	5.0	0.3	**6.5**	–

[a] Resistance ratio = IC_{90} drug resistant line/IC_{90} parent line for each drug. The IC_{90} values for the parental cell lines to each antitumor AA as expressed in micromolar units are: (1) SCC-25: 15, CDDP; 60, PAM; 24, 4-HC; 18, HN2; 295, BCNU; 180, THIO; (2) MCF-7: 40, CDDP; 15, PAM; 35, 4-HC; 2.5, HN2; 355, BCNU; 140, THIO; (3) SW2: 15, CDDP; 18, PAM; 120, 4-HC; 22, HN2; 120, BCNU; 50, THIO; (4) SL6: 60, CDDP; 45, PAM; 220, 4-HC; 4.5, HN2; 250, BCNU; 35, THIO; (5) G3361: 65, CDDP; 50, PAM; 380, 4-HC; 25, HN2; 250, BCNU; 150, THIO

their human counterparts and, therefore, that studies employing these in vitro models may have clinical relevance.

Sublines of each of the five parent human tumor cell lines with resistance to various antitumor AAs were developed using escalating selection pressure [44]. Cloned cell lines were selected from the surviving populations based on two criteria: (1) resistance to the exposure agents and (2) generation time similar to that of the parental cell lines. AA resistance in the cloned cell lines was stable for up to 3 months in most of the lines, although some lines have more stable resistance [46, 47]. Survival curves for each of these 25 AA-resistant cell lines were generated to the selecting agent and to various other AAs. Resistance ratios were calculated from the IC_{90} values obtained for the resistant sublines to each drug as compared with the IC_{90} value found for the parental cell lines to the same drug. These resistance ratios were used to determine the cross-resistance and collateral sensitivity of the AA-resistant cell lines as compared with the parental cell lines to each antitumor AA (Table 2). A resistance ratio of 1.00 indicates drug sensitivity equal to that of the corresponding parental cell line. Resistance ratios of 2.3 or greater are statistically significant at the $P < 0.05$ level as determined by comparison of the slopes of the survival curves generated for several representative examples. Resistance ratios of less than 0.7 indicate that the resistant cell line is significantly more sensitive to the challenging agent than is the parental cell line. For each AA and for each cell line to which resistance was developed (five cell lines resistant to each drug) there are approximately 25 observations of drug response (Table 2). Therefore, the denominators in the subsequent analysis of these data are 19–25.

In Table 3 the number of occurrences of cross-resistance and collateral sensitivity for each AA-resistant cell line are grouped by selecting agent. Cell lines resistant to CDDP infrequently exhibited changes in sensitivity to the other AAs examined (Table 3). Thus, cross-resistance was seen in 3 of 24 cases and collateral sensitivity, in 2 of 24 cases. The SW2/CDDP cell line was 5 times more sensitive to 4-HC than was the SW2 parent cell line at the IC_{90}. On the other hand, cells selected for resistance to PAM were frequently cross-resistant to the other AAs (12 of 21 determinations). Collateral sensitivity occurred most frequently in cell lines selected for resistance to 4-HC (10 of 21 determinations). Six examples of survival curves

Table 3. Summary by selection drug of the number of occurrences of cross-resistance and collateral sensitivity in the AA-resistant human tumor cell lines[a]

Cell lines	Number of occurrences[b]				
	Cross-resistance		Neutral	Collateral sensitivity	
	Major	Moderate		Moderate	Major
SCC-25/CDDP		2	3		
MCF-7/CDDP		1	4		
SW2/CDDP			3		1
SL6/CDDP			5		
G3361/CDDP			4	1	
Total CDDP		3 (3/24)	19	1	1 (2/24)
SCC-25/4-HC	1			1	2
MCF-7/4-HC			5		
SW2/4-HC			2	1	1
SL6/4-HC			3		1
G3661/4-HC				3	1
Total 4-HC	1	(1/21)	10	5	5 (10/21)
SCC-25/PAM	2	1	1		
MCF-7/PAM	3	1			1
SW2/PAM	2				2
SL6/PAM	1	1	2		
G3661/PAM	1		1	1	1
Total PAM	9	3 (12/21)	4	1	4 (5/21)
SCC-25/HN2			3	1	1
MCF-7/HN2		1	4		
SW2/HN2	1		2		1
SL6/HN2	1		3		
Total HN2	2	1 (3/18)	12	1	2 (3/18)
SCC-25/BCNU	2		1		1
MCF-7/BCNU	3		2		
SW2/BCNU	1		1	1	1
SL6/BCNU			4		
G3661/BCNU	2				2
Total BCNU	8	(8/21)	8	1	4 (5/21)

[a] Definitions of major and moderate cross-resistance and collateral sensitivity are given in Materials and methods

[b] Numbers in parentheses indicate the number of occurrences of cross-resistance or collateral sensitivity/the total number of determinations made

from which the data in Tables 2 and 3 were derived are shown in Fig. 2. The survival curves generated for the parental SCC-25 squamous carcinoma of the head and neck cell line, the parental SW2 small-cell lung-cancer cell line, and the parental SL6 non-small-cell carcinoma cell line upon exposure to various concentrations of PAM are shown along with those plotted for the corresponding PAM-resistant sublines and 4-HC resistant sublines following exposure to various concentrations of PAM. The response of the PAM-resistant sublines differs from that of the parental cell lines at $P < 0.0057$, $P < 0.027$, and $P < 0.0002$ for the SCC-25, SW2, and SL6 lines, respectively. The response of the 4-HC-resistant sublines to PAM differs from that of the parental cell lines at $P < 0.0015$, $P < 0.017$, and $P < 0.0025$ for the SCC-25, SW2, and SL6 lines, respectively.

Table 4. Summary of the occurrence of cross-resistance in the AA-resistant cell lines grouped by selecting agent

Resistant cell lines[a]	Number of occurrences of cross-resistance				
	Major	Moderate	None or minimal	Total	(%)
CDDP	0	3	21	3/24	(13%)
4-HC	1	0	20	1/21	(5%)
PAM	9	3	9	12/21	(57%)
HN2	2	1	15	3/18	(17%)
BCNU	8	0	13	8/21	(38%)
Total	20	7	78	27/105	(25%)

[a] There are five cell lines resistant to each AA

In Tables 4 and 6 the observed cell frequencies are small and, hence, exact tests of association were used throughout. The Kruskal-Wallis exact test for ordered categorical data [1, 26] was used to compare the distributions of cell lines (CDDP, 4-HC, PAM, HN2, and BCNU; Table 4) among the categories of occurrence of cross-resistance (major, moderate, and minimal). A similar test was conducted to compare cell line distributions among the categories of occurrence of collateral sensitivity (major, moderate, and minimal; Table 6). In Table 4 the distribution of the five resistant cell lines among the categories of occurrence of cross-resistance was found to be significantly different ($P < 0.0001$). In Table 6 the resistant cell lines were collapsed into three or more less homogeneous populations comprising CDDP and 4-HC versus the rest. The three population distributions differed significantly among the categories of occurrence of collateral sensitivity ($P = 0.026$). Closer inspection shows that the cell lines' distribution of CDDP, 4-HC, and HN2 are jointly significantly different from that of PAM among the categories of occurrence of cross-resistance at the 5% level (Bonferroni multiple-comparison adjustment) [41]. Similarly, CDDP and 4-HC individually differed from BCNU but cannot be declared jointly significantly different from BCNU. In Table 6, only CDDP differed significantly from 4-HC ($P < 0.05$).

When the occurrences of cross-resistance for the five human tumor cell lines resistant to each AA were totaled, the results shown in Table 4 were obtained. Major cross-resistance to other AAs for the cell lines rendered resistant to PAM was common. Overall, 57% of the PAM-resistant cell-line studies exhibited cross-resistance to other AAs. The corresponding figure for BCNU was 38%. For the other selecting agents (CDDP, 4-HC, and HN2), cross-resistance to other AAs occurred in 5%–17% of the studies. These differences were highly significant ($P < 0.01$; Table 4).

To determine the frequency of cross-resistance to specific AAs, the analysis shown in Table 5 was performed. For all 27 instances of AA cross-resistance, cross-resistance to HN2 occurred most frequently (9/27 cases). Cross-

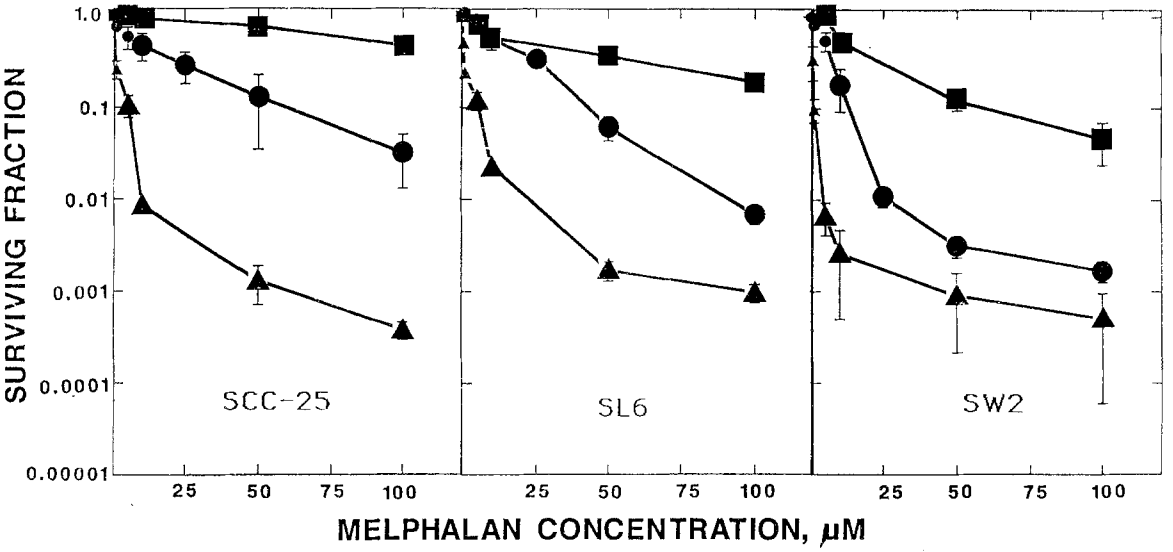

Fig. 2. Survival of SCC-25, SW2, and SL6 parental human tumor cells (●); SCC-25, SW2, and SL6 sublines resistant to PAM (■); and SCC-25, SW2, and SL6 sublines resistant to 4-HC (▲) after exposure to various concentrations of PAM for 1 h. The results are presented as the mean values ± SEM for three experiments

Table 5. Summary of cross-resistance occurrences by cell type and selecting agent[a]

Cell line	Selecting agent					Number of occurrences of resistance
	CDDP	PAM	4-HC	HN2	BCNU	
SCC-25	P,HC	HN2,HC, cP	HN2		HC,HN2	8
MCF-7	T	cP,HC, HN2,T		cP	cP,P HN2	9
SW2		cP,HN2		cP	HN2	4
SL6		HN2,HC		cP		3
G3661		HN2			P,HC	3
Total occurrences of cross-resistance	3	12	1	3	8	27[b]

[a] Symbols in columns are the agents to which the AA-resistant cell line is cross-resistant: T, THIO; B, BCNU; cP, CDDP; P, PAM

[b] Agents involved in the 27 instances of cross-resistance include HN2, 9; cP, 7; 4-HC, 6; PAM, 3; THIO, 2; BCNU, 0

resistance to CDDP was second in frequency, occurring in 7 instances. Third in frequency was resistance to 4-HC, which occurred in 6 instances, whereas resistance to PAM occurred 3 times and resistance to THIO, twice. We observed no instance of significant cross-resistance to BCNU. The cell lines in which resistance was developed to PAM as the selecting agent displayed 12 instances of cross-resistance. In fact, all 5 of the PAM-resistant cell lines were cross-resistant to HN2 and 3 of the 5 were cross-resistant to either 4-HC or CDDP. Thus, cross-resistance to HN2 occurred in the cell lines in which resistance was developed to PAM 100% of the time, whereas cross-resistance to BCNU in these cell lines corresponded to zero. Cell lines for which BCNU was the selecting agent had 8 occurrences of cross-resistance to other AAs, and 3 of 5 of these cell lines were cross-resistant to HN2. An

Table 6. Summary of the occurrence of collateral sensitivity in the AA-resistant cell lines grouped by selecting agent

Resistant cell lines[a]	Number of occurrences of cross-resistance				
	Major	Moderate	None or minimal	Total	(%)
CDDP	1	1	22	2/24	(8%)
4-HC	5	5	11	10/21	(48%)
PAM	4	1	16	5/21	(24%)
HN2	2	1	15	3/18	(17%)
BCNU	4	1	16	5/21	(24%)
Total	16	9	80	25/105	(24%)

[a] There are five cell lines resistant to each AA

Table 7. Summary of collateral sensitivity occurrences by cell type and selecting agent[a]

Cell line	Selecting agent					Number of occurrences of sensitivity
	CDDP	PAM	4-HC	HN2	BCNU	
SCC-25			P,cP,B	P,B	P	6
MCF-7		B				1
SW2	HC	HC,B	B,P	HC	HC,P	8
SL6			P			1
G3661	B	HC,cP	HN2,P B,cP		cP,HN2	9
Total occurrences of collateral sensitivity	25[b]	2	5	10	3	5

[a] Symbols in columns are the agents to which the resistant cell lines were collaterally sensitive: P = PAM; cP = CDDP; B = BCNU; HC = 4-HC.

[b] Agents involved in the 25 instances of collateral sensitivity include: PAM, 7; BCNU, 7; 4-HC, 5; CDDP, 4; HN2, 2

Table 8. Summary of the percentages of frequency of cross-resistance and collateral sensitivity for each of the five AAs[a]

AAs							
Producing cross-resistance		Cross-resistant to	collateral	Producing sensitivity		Collateral to	
PAM	57%	HN2	33%	4-HC	48%	PAM	28%
BCNU	38%	CDDP	26%	BCNU	24%	BCNU	28%
HN2	17%	4-HC	22%	PAM	24%	4-HC	20%
CDDP	13%	PAM	11%	HN2	17%	CDDP	16%
4-HC	5%	BCNU	0%	CDDP	8%	HN2	8%

[a] With respect to the therapeutic quality of the AA, columns 1 and 2 are negative properties and columns 3 and 4 are positive properties

examination of these results by cell type shows that cross-resistance to other AAs occurred with highest frequency in the MCF-7 human breast carcinoma cell lines, where 3 of the 5 lines were cross-resistant to CDDP and 2 of the 5 lines were cross-resistant to HN2 or THIO.

A summary of the number of occurrences of collateral sensitivity to other AAs observed for the various antitumor AA-resistant cell lines grouped by selecting agents is shown in Table 6. Collateral sensitivity occurred in 25 of the 105 determinations made. Nearly half of the AA-resistant cell lines were collaterally sensitive to 4-HC. Collateral sensitivity to 4-HC occurred at a statistically significantly greater frequency than did that to any of the other AAs tested in these studies (48% vs $\leq$24%; P <0.01).

The frequency of collateral sensitivity as analyzed by cell type and selecting agent is shown in Table 7. Collateral sensitivity occurred most frequently in the AA-resistant G3611 human melanoma and SW2 human small-cell lung-cancer cell lines. All 4 of the AA-resistant SW2 cell lines were collaterally sensitive to 4-HC (the cell line in which resistance developed was excluded from this analysis). Similarly, 2 of 4 of the AA-resistant SW2 cell lines were collaterally sensitive to PAM and 2 were collaterally sensitive to BCNU. The G3361 human melanoma cell line also had a high frequency of collateral sensitivity, with 3 of the 4 cell lines being collaterally sensitive to CDDP. Collateral sensitivity occurred most frequently in the cell lines where 4-HC was used as the selecting agent. In the 4-HC-resistant cell lines there were 4 instances of collateral sensitivity to PAM, 3 instances of collateral sensitivity to BCNU, 2 instances of collateral sensitivity to CDDP, and 7 instances of collateral sensitivity to HN2. Overall, collateral sensitivity developed most frequently to PAM and BCNU (7 instances each). There were 5 instances of collateral sensitivity to 4-HC, 4 instances of collateral sensitivity to CDDP, and 1 instance of collateral sensitivity to HN2.

Discussion

Preclinical modeling for the selection of chemotherapeutic agents for clinical trial has been a major challenge in cancer research [4]. After a long period of emphasis on in vivo systems (transplanted tumors in mice), the NCI screening program has evolved to the use of human tumor cell lines [5]. This is reasonable since human tumor cells, particularly those that exhibit differentiation features consistent with the cell of origin, might be expected to have biochemical properties and targets similar to their in vivo counterparts. In the present study, it was reassuring that the sensitivity of the five parental human tumor cell lines to the various antitumor AAs correlated closely with clinical treatment results (Table 1). Thus, melanoma is generally unresponsive to AAs. Small-cell lung cancer, breast cancer, and squamous cell carcinoma of the head and neck are relatively responsive [10]. Within tumor cell types there was also reasonably good correlation with specific AA activity. For example, CDDP is clearly a clinically superior drug for head and neck cancer, and we found that the parental SCC-25 cell line was exquisitely sensitive to CDDP in vitro (Fig. 1). The observations and comparisons made in this report are, at best, semiquantitative and should not be overemphasized. Nevertheless, they are reassuring and suggest that our preclinical in vitro observations may have important clinical relevance [28, 55].

In addition to dose, the use of agents in combination has been central to the development of curative regimens [18]. An important criteria for the selection of agents for use in combination has been lack of cross-resistance. Examination of the cross-resistance patterns of the various AA-resistant cell lines indicated that for the CDDP-, 4-HC-, and HN2-resistant human tumor cell lines, cross-resistance to the other AAs was uncommon (5%–17% of the cases examined). In contrast, the PAM-resistant human tumor cell lines exhibited major cross-resistance to other AAs in almost 60% of cases (P <0.01). The ranking order of the agents to which the AA-resistant cell lines were most frequently cross-resistant was HN2 > CDDP > 4-HC > PAM > THIO > BCNU. This would suggest that if AAs are to be used in sequence, PAM should not be selected as the initial agent. On the other hand, PAM was highly active in cell lines resistant to other AAs.

Clinical data relating to the problem of cross-resistance among the antitumor AAs is limited. The generalization has been that prior exposure to an AA decreases responsiveness to subsequent treatment not only with that same drug but also with other AAs. This generalization is not limited to AAs but can be made for essentially all chemotherapeutic agents and may well relate to the clonal evolution to heterogeneity that is intrinsic to progressive cancer. The only human tumor that is treated primarily with PAM (in combination with prednisone) is myeloma. The majority of myeloma patients respond initially to PAM and will, when retreated, respond again, although the duration of response to secondary treatment is much shorter [2]. Other antitumor AAs such as CPA may produce responses in myeloma after PAM treatment, but these responses tend to be incomplete and much shorter in duration than those obtained in AA-naive patients [2]. A 10- to 12-fold increase in the dose of PAM (high-dose marrow protection) will regularly produce responses even in patients whose disease is refractory to standard doses of PAM [2]. These clinical observations are consistent with our in vitro findings. The above-mentioned generalizations with respect to myeloma response to AAs hold for other neoplasms, al-

though experience with the latter is more limited and the use of agents in combination makes interpretation of the data difficult [15].

One potentially broad strategy to improve the response to chemotherapy is to treat patients initially with one effective agent until they develop resistance to that agent, which leads to increased responsiveness to a second agent – so-called collateral sensitivity [28, 55]. Collateral sensitivity occurred in 25 of the 105 survival-curve determinations in the AA-resistant human tumor cell lines studied (Table 6). In all, 16 of these occurrences were major, i.e., a 3- to 10-fold increase in sensitivity at the IC_{90} was observed to the challenging agent as compared with the parental cell line's sensitivity to the same drug (Table 6, Fig. 2). Given the steepness of the dose/concentration response/survival curves generated for the AAs, this effect could have profound therapeutic implications [15]. Collateral sensitivity occurred significantly more frequently in the 4-HC-resistant cell lines (48%) than in the cell lines selected for primary resistance to other selecting agents (8%–24%). This finding implies that 4-HC (and presumably CPA itself) may best be used early in treatment because prior exposure to this AA might lead to an increased response to other antitumor AAs. The ranking order of the challenging agents involved in the 25 instances of collateral sensitivity was PAM = BCNU > 4-HC > CDDP > HN2. Thus, exposure to PAM in particular, but to BCNU as well, produced resistance that crossed to other antitumor AAs with high frequency (Table 4). On the other hand, PAM and BCNU proved to be good agents for the treatment of the AA-resistant cell lines. If this observation extrapolates to the clinical setting, it would mean that PAM and BCNU are inferior to the other antitumor AAs as first-order treatment but are superior for second-order treatment.

Studies of AA-resistant tumor cell lines have established that multiple mechanisms can contribute to resistance to antitumor AAs, including: (1) loss of capacity for transport across the cell membrane; (2) conjugation and inactivation in the cytoplasm by the GST/GSH system or by metallothionein; (3) an increase in aldehyde dehydrogenase, which inactivates CPA; and (4) enhanced repair of AA-induced lesions in DNA [6, 23, 27, 45, 46, 50, 51, 53, 56]. One or more of these mechanisms have been described for the different AA-resistant cell lines. This multiplicity of resistance mechanisms is consistent with the observation that cross-resistance among the antitumor AAs is, in general, uncommon. Multifactorial resistance, that is, two or more mechanisms operative in the same cell line, has been described and is probably common [46, 47, 51]. The finding that low levels of cross-resistance are relatively common (Table 2) is consistent with the notion that multifactorial resistance is common.

Several of the above-mentioned resistance mechanisms are specific for individual AAs. For example, increased aldehyde dehydrogenase activity as a mechanism of resistance is specific for CPA and other oxazaphosphorines [40]. Such a mechanism would not be expected to cross to the other antitumor AAs, which is consistent with our finding that cells selected for resistance to 4-HC were infrequently cross-resistant to other AAs. Decreased membrane transport occurs as a mechanism of resistance for PAM and HN2 and may be specific for each of these drugs [21, 22, 32, 49]. This observation is consistent with our finding few cases of cross-resistance to HN2. However, the frequency of cross-resistance in the PAM-resistant human tumor cell lines indicates that a more general mechanism of resistance must also be operative in these tumor lines. The BCNU-resistant human tumor cell lines demonstrated the second highest frequency of cross-resistance (Table 3). The GSH/GST system, especially the μ isozymes of GST, has been implicated in BCNU resistance [53]. DNA repair is complex, but at least in the case of BCNU, removal of the monoligand by *O*-6-methylguanine methyltransferase is specific to this drug [29, 38]. Our data are consistent with this interpretation, but the heterogeneity of cross-resistance patterns in the BCNU-resistant cell lines suggests that other mechanisms must be operative as well.

The observation that frequent cross-resistance occurs in tumors made resistant to PAM has been corroborated in vivo in the P388 leukemia cell line [52]. The P388/L-PAM tumor line was cross-resistant to approximately half of the antitumor agents tested against it, including 6 of 10 (60%) alkylating agents. The P388/L-PAM tumor line was also frequently cross-resistant to DNA-binding agents but was not cross-resistant to antimetabolites. On the other hand, similar to our findings in the AA-resistant human tumor cell lines, the P388/CPA tumor line was cross-resistant to only 4 of the 12 (30%) alkylating agents tested. The P388 line was as sensitive as the parent P388 tumor line to antimetabolites and DNA-binding agents [52].

In conclusion, resistance to AAs is more difficult to produce than is resistance to other antitumor agents and, even after extensive dose escalation, can be produced only at relatively low levels. The cell lines resistant to HN2, CDDP, and 4-HC exhibited cross-resistance in 5%–17% of studies, whereas for cell lines resistant to PAM and BCNU, cross-resistance occurred in 57% and 38% of studies, respectively (Table 8). The agents against which this cross-resistance was developed were most commonly HN2, CDDP, and 4-HC, respectively. Collateral sensitivity, a feature with positive therapeutic implications, occurred with unanticipated frequency particularly in cell lines resistant to 4-HC. PAM, which most commonly produced cross-resistance to other antitumor AAs (an adverse quality), was the agent against which other AA-resistant cell lines developed collateral sensitivity. Overall, 4-HC had the most favorable qualities in this study of antitumor AAs in that as a selecting agent, it infrequently produced cross-resistance to other antitumor AAs and most commonly produced collateral sensitivity. Therapeutically, therefore, PAM may have properties that make it unfavorable as an initial treatment agent, whereas 4-HC and, perhaps, the chemically related CPA have properties that may make them better first-line treatment agents.

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