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ORIGINAL ARTICLE

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Double high-dose chemotherapy with stem cell rescue (HD-SCR) in patients with breast cancer – effect of sequence

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Abstract *Introduction*: A preliminary analysis of our double high-dose chemotherapy with stem cell rescue (HD-SCR) clinical trial for breast cancer, and preclinical cross-resistant studies, suggested that melphalan (M) adversely affected response to subsequent chemotherapy, i.e., that the sequence of alkylating agents (AAs) might affect response. We, therefore, constructed and examined preclinical models to determine whether prior exposure to M, in fact, adversely affected response to other therapy. *Purpose*: The purpose of the study was to determine whether the sequence of AAs, specifically the prior use of M, adversely affected response to subsequent treatment. *Methods*: The methods employed were the following: (1) Human tumor cell lines rendered resistant by in vitro sequential exposure to five different AAs were developed. The resistant cell lines were examined for cross-resistance to alkylating and other agents. (2) In vivo studies in the p388 mouse leukemia for resistance and cross-resistance among the AAs. (3) In vivo studies of the effect of sequence of AAs on response in mice bearing EMT6 breast cancer. (4) The double transplant model was developed in the mouse and the sequence of high-dose AAs was studied. (5) Biochemical and reverse transcriptase-polymerase chain reaction (RT-PCR) studies of the various resistant tumor cell lines. *Results*: (1) The in vitro human tumor cells resistant to M were cross-resistant in 57% of tests to other AAs. In contrast, resistance for other AAs crossed to other agents in only 10 to 20% of tests. (2) The in vivo studies of p388 indicated that resistance to M commonly crossed to other AAs and many non-AAs. (3) The results for the mouse breast cancer (EMT6) studies of the sequence of AAs again indicated that M employed first markedly reduced responsiveness to subsequent treatment, particularly with AAs. (4) The double transplant model: again, M first markedly reduced response to other agents. (5) The in vitro resistant human tumor cell lines, particularly the breast cancer cell line MCF7, were found to contain high concentrations of glutathione S1 transferase gamma, which is consistent with that mechanism being responsible for resistance. *Conclusion*: The sequence of alkylating agent treatment may substantially influence response. Melphalan, particularly, produces resistance that commonly crosses to the other AAs. Mechanistic studies indicate significant changes in glutathione S1 transferase, a known mechanism for broadly based resistance to AAs.

Key words Alkylating agents · Melphalan · Resistance

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Introduction

High-dose chemotherapy, largely with alkylating agents (AAs), and with stem cell rescue (HD-SCR) for breast cancer was a major and markedly newsworthy item at the last American Society for Clinical Oncology Plenary Session [1–11]. With one exception, the comparative studies did not show improved survival for the high dose arm. The tentative conclusions were that the high-dose arm improved the complete response rate and may have provided an up to 20% improvement in disease-free survival beyond 3 years. In the high risk adjuvant setting (10+ positive nodes), the studies were not positive for improved survival, though suggested evidence of a diminished relapse rate was observed in one study, and toxicity was a major problem affecting death in 7% of patients in another study. There was general agreement that these analyses were too early and further follow up

will be required to define the activity of these programs [1–11].

The rationale for high-dose combination chemotherapy for solid tumors has been presented [12–18]. In general, the addition of a second or third agent for HD-SCR can be accomplished without significant compromise in dose. However, the addition of a fourth agent has not been possible beyond phase I studies because of toxicity. This is a limitation, given the fact that several new and promising agents that might improve the program are available. Another approach that would permit additional agents would be a second or double course of HD-SCR therapy. This has been done employing the same agents as in the first course, or, alternatively, different agents are employed in the two courses with the anticipation and hope that there will be some lack of cross-resistance [12–18].

Because of the above, we initiated a clinical trial of double or tandem transplant which included remission induction with an Adriamycin-based combination followed by a double transplant to include cyclophosphamide, thiotepan, carboplatin (CTC) and melphalan (M)/Taxol. In examining some of our previous and ongoing studies in the laboratory of resistance to AAs, we observed that M much more commonly produced cross-resistance to AAs generally than did the other AAs [14, 15]. We, therefore, extended our preclinical studies and clinical reviews of the issue as to whether the sequence of AAs, particularly the use of M first, would affect overall response. In this paper, we present biological and biochemical evidence that the sequence of alkylating agent administration influences response. In a companion paper, we analyze the effect of the interval on response [19].

Methods and results

We have studied the effect of sequence in the use of AAs in five systems: (1) resistance and cross-resistance in human tumor cell lines; (2) cross-resistance among the AAs – in vivo studies; (3) in vivo studies of the sequence of AAs; (4) the mouse double (double) transplant model, and (5) biochemical studies of the sequence effect.

Human tumor cell lines. In vitro resistance and cross-resistant studies

The methodology has been published [12, 14, 15]. Five human tumor cell lines including breast carcinoma (MCF7), head and neck squamous cell carcinoma (SCC25), non-small cell lung carcinoma (SL6), small cell lung carcinoma (SW6), and melanoma (G3361) were selected for study primarily because they showed evidence of differentiation similar to their clinical counterpart and because the pattern of response to antitumor agents was reasonably consistent with response in the clinic. Resistance was produced in vitro by serial passage in the presence of increasing concentrations of the selecting agent. Resistance to the AAs was difficult to produce, taking up to 12 months. Resistance was low level, that is, three- to eightfold and was semi-stable [12, 14, 15].

The definitions of resistance, cross-resistance, resistance ratios, and collateral sensitivity are summarized in Table 1. The resistance ratios for the 25 cell lines are presented in Table 2 (taken from [15]) and the results are summarized in Table 3. Note in Table 2 that low levels of cross-resistance are relatively common.

Resistance was defined arbitrarily as a RR (resistance ratio) of >3× (i.e., an IC90 at least threefold greater than the IC90 in the parent line). Major cross-resistance as presented in Table 3 was defined as an IC90 of >2/3 the resistance ratio of the resistant line (Table 1). Moderate cross-resistance was defined as a resistance ratio of >1/3 and <2/3 the RR of the resistant cell line.

For M, cross-resistance, usually of a major degree, occurred to the other AAs in 57% of the 21 tests (Table 3). For the other AAs, cross-resistance occurred in 5%, 8%, and 18% for 5-OH-cyclophosphamide (5HC), CDDP, and HN2, respectively. Bis-chlorethylnitrosourea (BCNU) was intermediate. Thus, exposure to M in contrast to the other AAs produced a frequent and major degree of cross-resistance. 4-Hydroxycyclophosphamide (4HC) produced collateral sensitivity to the other AAs in almost 50% of tests. For the other agents, including M, collateral sensitivity was obtained in approximately 25% of tests. Our data would suggest that 4HC is a good drug to use first since it induced a minimum degree of cross-resistance, and a significant degree of collateral sensitivity. M, in accordance with these observations, would be the wrong drug to use first.

Cross-resistance among the alkylating agents – in vivo studies

The methodology for these studies has been published [13, 16, 17, 18]. P388 is a mouse lymphoblastic leukemia, which has been extensively studied and was employed for a time as the primary screen for the selection of antitumor agents for clinical trial by the National Cancer Institute. The numbers in Table 4 represent the log tumor cell kill for an LD10 dose of AAs. Thus, a −7 = a 7-log kill. The log kill of an LD10 dose for the various AAs was com-

Table 1 Definitions of drug resistance, cross-resistance, and collateral sensitivity

IC90 (inhibitory concentration; IC)	Concentration that produces a 90% inhibition of growth as measured by cell numbers and/or colony formation
Ca	Cancer cell line
Ca(0)	Sensitive, "wildtype" or parent cell line
Ca(A)	Resistant to drug A
Resistance ratio (RR) or fold resistance	IC90 CaA(A)/IC90 Ca(0)
Cross-resistance	Cell line resistant to drug A is also resistant (cross-resistant) to drug B

Example drug tested	Tumor cells	IC90	Resistance ratio	Interpretation
A	Ca(0)	10 μ*M*	1	
A	Ca(A)	50 μ*M*	5	5× Resistant
B or	Ca(0)	20 μ*M*	1	
B (see text)	Ca(A)	20 μ*M*	1	No cross-resistance
or B	Ca(A)	80 μ*M*	4	4× Cross-resistant
Collateral sensitivity (hyperresponsive)				
B	Ca(A)	2 μ*M*	0.1	Collateral sensitivity

Table 2 Resistance ratios of the alkylating agents (*AAs*) resistant human tumor cell lines to five AAs[a]. *IC*90 concentration that produces a 90% inhibition of growth as measured by cell numbers and/or colony formation, *M* melphalan, *CDDP* cisplatin, 4-*HC* 4-hydroxyphosphamide, *HN*2 nitrogen mustard, *BCNU* bis-chloroethylnitrosourea

Antitumor AAs Cell lines	CDDP	M	4-HC	HN2	BCNU
Squamous carcinoma					
SCC-25	1.0	1.0	1.0	1.0	1.0
SCC-25/CDDP	30.0	5.0	3.0	1.8	2.0
SCC-25/2HC	2.7	5.5	3.6	6.3	1.1
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
MCF-7/CDDP	6.5	2.0	1.1	1.6	1.4
MCF-7/M	4.9	7.0	5.0	5.6	0.2
MCF-7/4-HC	1.3	1.0	9.0	2.0	1.1
MCF-7/HN2	2.3	0.8	1.3	5.5	0.7
MCF-7/13CNU	4.0	4.3	1.3	20.0	2.7
Small cell lung cancer					
SW2	1.0	1.0	1.0	1.0	1.0
SW2/CDDP	3.3	0.7	0.2	1.0	1.8
SW2/M	6.7	3.3	0.3	2.5	0.3
SW2/4-HC	1.3	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
SL6/CDDP	3.5	1.3	1.5	1.5	1.9
SL6/M	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	1.0	0.5	0.3	3.0	4.2
Melanoma					
G3361	1.0	1.0	1.0	1.0	1.0
G3361/CDDP	9.2	2.0	1.5	1.7	0.6
G3361/M	0.4	4.0	0.1	5.0	0.8
G3361/4-HC	0.5	0.5	5.4	0.3	0.4
G3361/HN2	–	–	–	–	–
G3361/BCNU	0.3	5.5	5.0	0.3	6.5

[a] Resistance ratio + IC90 drug resistant line/IC90 parent line for each drug. The IC90s for the parental cell lines to each antitumor AA in micromolar are:
1. SCC-24: 15, CDDP; 60, M; 24,4-HC; 18, HN2; 295, BCNU; 180, THIO
2. MCF-7: 40, CDDP; 15, M; 25,4-HC; 2.5, HN2; 355, BCNU; 140, THIO
3. SW2: 15, CDDP; 18, M; 120,4-HC; 22, HN2; 120, BCNU; 50, THIO
4. SL6: 60, CDDP; 45, M; 220,4-HC; 4.5, HN2; 250 BCNU; 35, THIO

parable and in the range of −7 (Table 4). No tumor cell kill was achieved in the M-resistant cell line and cross-resistance obtained for five of the seven agents studied. Indeed, four of the five non-AAs were cross-resistant to M (Table 4). These data are summarized in Table 5 where the major degree of cross-resistance to M-resistant cell lines contrasts with the relative lack of cross-resistance for the other cell lines [16, 17].

In vivo studies of the sequence of alkylating agents

In order to more precisely model our HD-SCR double approach, we developed the preclinical in vivo model schematically presented in Fig. 1 [18]. One million mouse EMT6 breast cancer cells were inoculated subcutaneously on day 0. By 5 days, the tumor had increased to an estimated 6.6 logs. At that time, the first AA treatment was delivered. Dose-response studies using the excision assay had been performed and the dose required to produce a 2.5 log kill was delivered. Thus, the log tumor cell kill for the first treatment was constant. Five animals were included in each test. Since the mice had to be killed for the excision assay, a separate group of animals was studied, and received the second course of treatment on day 12 or day 17. The excision assays on days 12 and 17 reflect the tumor cell kill of that dose as affected by prior treatment on day 5 and the degree of recovery by day 12 and day 17 [18].

The effect of the sequence of treatment is presented graphically in Fig. 2 and in tabular form in Table 6. Note the threefold greater tumor cytoreduction (log kill) for the second treatment when the first treatment is cyclophosphamide (CPA) compared with M. One interpretation is that the tumor cells treated first with M were much more resistant 12 days later than those whose first treatment was CPA. By day 17, there is slight recovery of the tumor first treated with M [18].

Mouse double transplant model

In order to mimic, i.e., model the clinical high-dose sequence situation as closely as possible, we developed an autologous (synergeneic) stem cell transplant model in the mouse and extended it to modeling of two sequential, i.e., double transplants (Table 7). This provided an opportunity to study sequence. Following inoculation, transplant was done on day 5 and day 12 (for details see Fig. 2). In this model, CPA at the dose given was clearly more active than M. However, in both circumstances where CPA was given first, the effect was disproportionately greater. And, CPA → M was far superior to M → CPA [18] (Table 7).

Biochemical and RT-PCR studies

The methodology for the biochemical and PCR studies has been published [19, 20]. The data are presented in Table 8. The first column delineates the five cell lines. Within each cell line, there is the parent cell followed by the corresponding resistant cell lines. The degree of resistance, i.e., "fold R" or "fold resistance", is presented in column 2. "One" ("1") represents the sensitivity/resistance of the parent line.

Where the increase is greater than fourfold, the numbers are presented in boldface type. Thus, for the breast cancer line, MCF7, resistance to M is associated with a total glutathione transferase (GST) elevation of 22.4-fold, most of which is the α isozyme (increase of 10.5-fold) and less so of the μ isozyme (3.8-fold). Metallothionein (MTα) is increased over fourfold in the MCF7, thiotepa, and BCNU cell lines, and approximately sixfold in the small cell lung cancer M- and HN2-resistant lines (SW2/M and SW2/HN2). Also, MT is increased fivefold in non-small cell lung cancer CDDP, HN2 resistant cell lines (SL6/CDDP and SL6/HN2; Table 8). γ-Glutamylcysteine synthetase (γ-GCS) heavy and light subunits form the heterodimeric enzyme, which is the rate-limiting step in de novo glutathione (GSH) biosynthesis. Generally, there was only a small increase in the expression of the light subunit in some of the resistant cells. DNA-dependent protein kinase (DNA-PK) is a trimeric enzyme complex with a role in the repair of double-strand DNA breaks. The catalytic subunit of this enzyme was expressed at twofold higher levels in M-, HC- and CDDP-resistant cells.

Discussion

The microenvironment of the common epithelial solid tumors presents therapeutic obstacles, such as poor blood supply, low growth fraction, and tumor hypoxia. These obstacles make it unlikely that a single HD-SCR

Table 3 The alkylating agents (*AAs*). Cross-resistance and collateral sensitivity. *CDDP*, *M* melphalan, *HN*2, *BCNU* bis-chloroethylnitrosourea

Resistant cell lines to:	Total	Cross-resistant to other AAs			Collateral sensitivity to other AAs		
		Total (%)	Major	Moderate	Total (%)	Major	Moderate
CDDP	24	2 (8%)	0	2	3 (13%)	1	2
41-1C	21	2 (5%)	1	1	10 (48%)	5	5
M	21	12 (57%)	10	4	5 (24%)	4	1
HN2	19	2 (18%)	2	0	4 (22%)	2	2
BCNU	21	8 (38%)	5	3	5 (23%)	4	1
Total	106	26 (25%)	18	10	27 (26%)	16	11

Table 4 Cross-resistance among the alkylating agents (*AAs*) in p388 mouse leukemia. Number = log tumor cell kill per LD10 dose. Spiromustine, tetraplatin, and pyrizine-azo-OH are non-classical AAs. *M* melphalan, *CPA* cyclophosphamide, *cPlat* cisplatin, *BCNU* bis-chlorethylnitrosourea, *AMSA*

p388 leukemia sensitive (*S*) and resistant (*R*) to		M		CPA		cPlat		BCNU	
		S	R	S	R	S	R	S	R
M		−7	0	−6	−7	−7	−7	−7	−7
CPA		−7	−7	−7	−1	−7	−7	−7	−7
cPlat		−7	−2	−3	−7	−6	+2	−3	−4
cPlat (repeat)	−7	+2	−5	−5	−6	+2	−7	−5	
BCNU	−7	−8	−7	−7	−7	−8	−7	+2	
Hepsulfam		−7	−1	−5	−6	−4	0	−4	−4
Spiromustine	−6	−2	−7	−5	−4	+1			
Tetraplatin		−4	0						
Pyrizine-azo-OH		−7	−1						
X-resistant/total		5/7		0/5		2/6		0/6	
Resistance to non-alkylating agents									
Doxorubicin		−7	−7	−5	−6	−7	−7		
Mitoxantrone	−6	−3			−3	−8			
AMSA		–	−2	−3	−6	−2	−7		
Merbarone		−7	−3			−5	−7		
Vincristine		−5	−1	−4	−4	−7	−7		
Penclomidine	−1	−2	−4	−1	−1	−1	−4	−1	
X-resistant/total		4/5		1/4		0/5		1/1	

Table 5 P388 Mouse leukemia. Summary of resistance/cross-resistance. *M* melphalan, *CPA* cyclophosphamide, *cPlat* cisplatin, *BCNU* bis-chloroethylnitrosourea, *AAs* alkylating agents

	Acquired resistance to:			
	M	CPA	cPlat	BCNU
Cross-resistance to:	5/7	0/5	2/6	0/5
Other AAs	4/5	1/4	0/5	1/1
Non-AAs				
Total	9/12	1/9 (15%)	2/11 (15%)	1/6 (15%)

treatment would be curative [18]. Major tumor cytoreduction with a first HD-SCR course may reduce these obstacles for the second HD-SCR course. Comparative studies of standard dose adjuvant chemotherapy in breast cancer indicate that no more than four to six courses of treatment are optimal [21]. Taken together, these data suggest that two to five HD-SCR courses may be required for optimal effect.

The double HD-SCR study of neuroblastoma trial has been reported as positive [17]. It is too early to evaluate the ongoing double studies of patients with breast cancer. Given the activity of M and Taxol at standard doses, there was reason to believe that high-dose MT followed by CTC might be superior to CTC only [8, 9, 10]. The early lack of evidence that a second HD-SCR course would produce a superior result, and that tumor cell lines resistant to M were widely cross-resistant, led us to examine and extend our preclinical models.

Studies in p388 mouse leukemia strongly support the position that the sequence of AAs, particularly M, may have a major effect on the therapeutic index [16, 17]. The sequential in vivo studies of the EMT6 breast cancer in mice indicate that a fixed cytoreductive dose of M adversely affects response to the second agent (Table 4). Some degree of recovery of cytotoxicity, i.e., loss of resistance, occurred over time, a subject that will be dealt with in a companion paper which deals with the interval between treatments [18a]. Similar results were seen in our mouse double or double HD-SCR studies (Table 7).

What are the biochemical mechanisms responsible for the resistance and cross-resistance patterns, particularly with respect to M? The high level of cross-resistance to other AAs of the M-resistant tumor cell lines suggests a mechanism common to the AAs. This is supported by the mechanism-based studies which indicate that for the breast cancer cell line, resistance to M is associated with a tenfold or greater increase in the α isozyme of GST and a 22-fold increase in total GST activity (Table 8) [22, 22a, 23]. Increases in thiol protective pathways have been implicated in many aspects of resistance to AAs. In particular, bifunctional nitrogen mustards such as

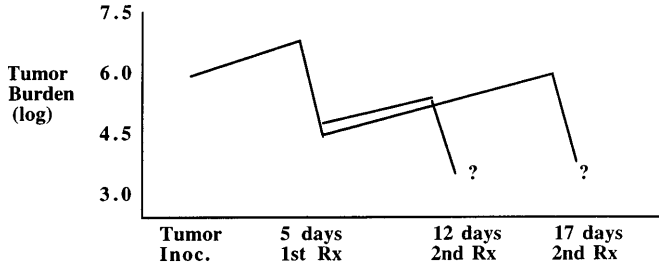

Fig. 1 Breast cancer sequence studies in the EMT-6 mouse model. One million tumor cells were inoculated subcutaneously. On day 5, the first treatment was delivered at a dose known to produce a 2.5- to 3-log tumor cell kill. On days 12 or 17, a second treatment was given across a dose-response curve. The animals were killed 24 h later and a cloning assay performed. The results are presented in Table 6 and Fig. 2

chlorambucil and M [22, 22a, 23] are substrates for the GSTα family of isozymes with Km values in the micromolar range. Other isoforms of GST can catalyze the conjugation reaction albeit with lower catalytic constants. Thus, it is possible that the increased expression of GSTμ in the M-resistant MCF7 may also be a contributory factor in the resistant phenotype. Our data on the other cell lines is limited. Total GST is increased in the small cell lung cancer lines (Table 8).

Metallothionein (MT) is increased in two of the MCF7 AA-resistant cell lines, and in essentially all of the small cell lung cancer cell lines and non-small cell lung cancer cell lines, and less impressively in melanoma (Table 8). MT is a small protein with multiple cysteine residues, which may combine with the electrophilic sites of AAs. Both GSTα and MT increase have been associated with AA resistance. M is an important agent, particularly for high-dose therapy, and the potential for increase in its activity in M-sensitive lines, and particularly the prevention and overcoming of M resistance, has major therapeutic implications. Ethacrynic acid is an inhibitor of GST in preclinical systems, but its diuretic potency has precluded clinical effectiveness. Newer peptidomimetic GST inhibitors have been described [24, 25, 26] and will be tested in our M-resistant lines.

Mechanisms of resistance that are specific to individual agents include plasma membrane transport for

Fig. 2 Effect of sequence of alkylating agents on response of mouse breast cancer (EMT6)

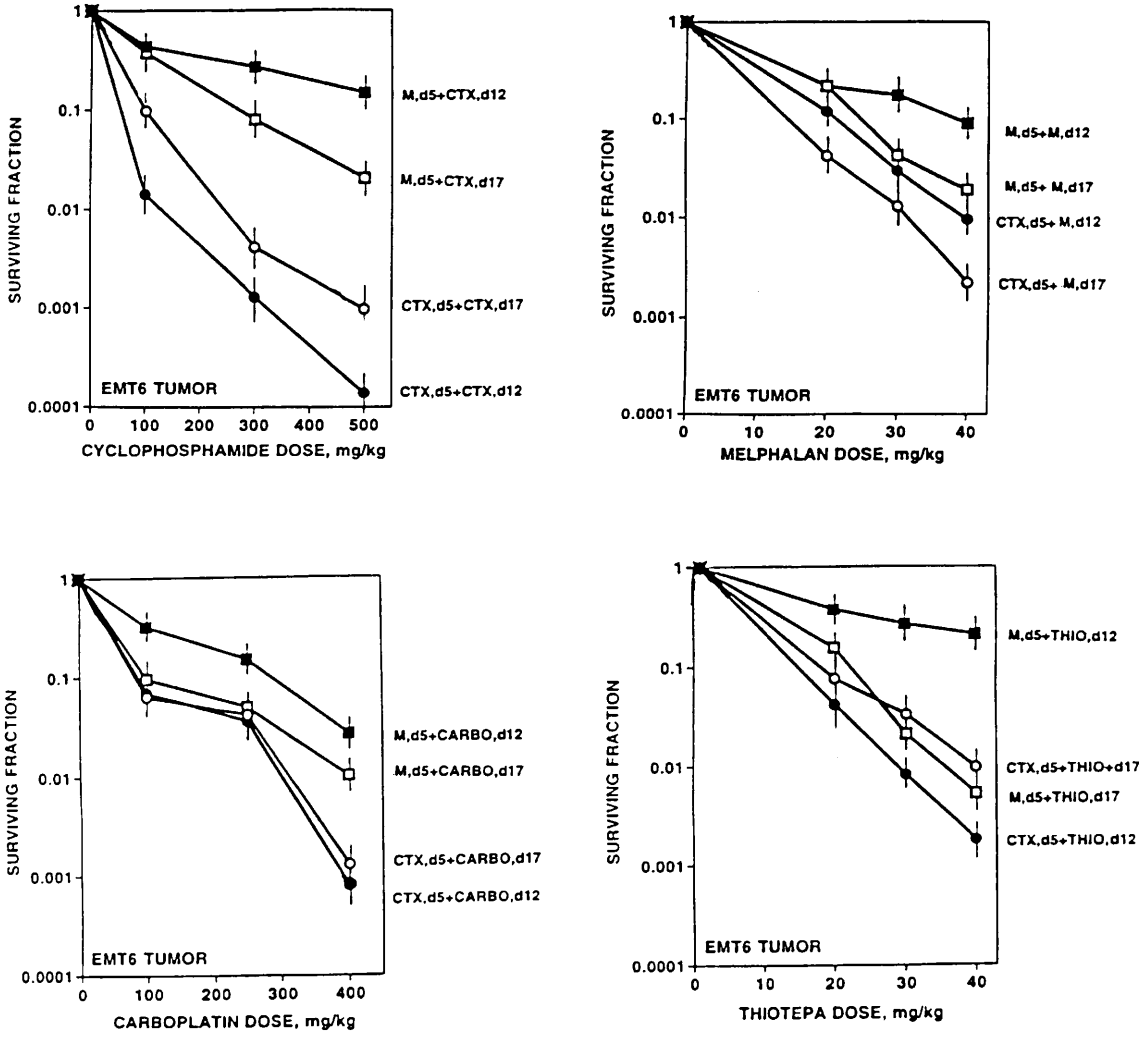

Table 6 EMT6 breast cancer in mice. Effect of sequence of alkylating agents. *M* melphalan, *CPA* cyclophosphamide, *TSPA* thiopeta, *carbo* carboplatin

	Log tumor cell kill		
	1st Rx (day 5)	2nd Rx	
		Day 12 or	Day 17
M → M	2.5	1.0	1.6
M → CPA	2.5	0.5	1.5
M → TSPA	2.5	0.2	1.5
M → carbo	2.5	1.5	1.9
Mean	0.8	1.6	
CPA → M	2.5	2.0	2.7
CPA → CPA	2.5	3.8	3.1
CPA → TSPA	2.5	2.7	2.2
CPA → carbo	2.5	3.0	2.9
Mean	2.7	2.7	
Taxol → M	2.5	0.8	1.7
TSPA → M	2.5	0.2	1.6

Table 7 Growth delay of the EMT6 murine mammary carcinoma after double transplant. *CPA* cyclophosphamide 400 mg/kg, *M* melphalan 30 mg/kg

Treatment group	Tumor growth delay (days)
M day 5	5.1 ± 0.4
M day 5 → M day 12	7.2 ± 0.7
M day 5 → CPA day 12	11.3 ± 1.4
CPA day 5	19.6 ± 1.4
CPA day 5 → M day 12	42.8 ± 2.3
CPA day 5 → CPA day 12	46.4 ± 2.0

nitrogen mustard and M, aldehyde dehydrogenase which converts OH-C (4-hydroxy-cyclophosphamide) to the inactive carboxy-C. The same is true for the closely related IFF (ifosfamide). DNA repair mechanisms may be specific, such as O6 methyl transferase for the nitrosoureas [27, 28, 29].

Table 8 Correlation between resistance and molecular pharmacology. *Fold R* IC50 resistant line/parent line, *GSH* glutathione, *MT* metallothionein, *GST* glutathione transferase; (alpha, mu, and pi are isozyme families of glutathione transferase). Human cell line: *SCC* squamous cell carcinoma of the head and neck; *MCF7* adenocarcinoma of the breast; *SCLC* small cell lung cancer; *NSCLC* non-small cell lung cancer; *CDDP* cisplatin; *4HC* activated cyclophosphamide; *M* melphalan; *TSPA* thiotepa; *BCNU* bischlorethylnitrosourea; *HN2* = nitrogen mustard, *PK* protein kinase, *ND* not done

					GST						
Cell line	Fold R	GSH (μ*M*/mg/prot)	MT (μ*M*/mg/prot)	Total (a)	Alpha (fold)	Mu	Pi	Gamma-GCS (heavy)	Gamma-GCS (light)	DNA-dependent PK	DNA (repair)
SCC25	1*	55	50	853			ND				
SCC25/CDDP	30	55	100	1760							
SW25/4HC	2.7	31		1264							
SCC25/M		20		826							
SCC25/HN2	2.3	28	57	845							
SCC25/BCNU	1.4	20		823							
MCF7	1	35	94	10	1	1	ND	1	1	1	
MCF7/HC	1.3	40	122	20	2.7	0.98	ND	0.86	1.5	2.37	
MCIF7/CDDP	6.5	98	238	7	1.4	1.2	ND	0.86	1.35	2.03	
MCF7/HN2	2.3	38									
MCF7/M	4.9	45		224a	10.5a	3.8a	ND	1	1.42	11.83	
MCF7/TSPA		83	421a	5	1.9	0.13	ND	0.95	0.81	0.97	
MCF7/BCNU		47	414a	15							
SCLC											
SW2	1	50	50	< 8							
SW2/CDDP	3.3	86	193	< 8							
SW2/4HC	1.3	129	144	168a							
SW2/M	6.7	174	300a								
SW2/HN2	3	244	348a	105a							
SW2/BCNU	1	315	275a	351							
NSCLC											
SL6	1		80	180							
SL6/CDDP	3.5	55	481a	278							
SL6/M	0.8	55	186	247							
SL6/HC	1.3	81									
SL6/HN2	2.3	141	469a	355							
SL6/BNCU	1										
Melanoma											
G336	1	50	87								
G336/CDDP	9.2	43	113								
G336/M	0.4	58	51								
G336/HC	0.5	44	155								
G336/BCNU	0.3	30	100								

* All 1s = parent cell line normalized to 1
a > 3× parent cell line

A major complication in identifying the mechanism responsible for resistance is the fact that resistance is commonly multifactorial, that is, two or more mechanisms contribute to the resistance [29]. While the increase in GSTα is perhaps quantitatively sufficient to explain the degree of biological resistance (4.9-fold), it is important to note that in addition to total GST and GSTα, there is also a consistent, if moderate, increase of GSH reflected by a similar mildly enhanced expression of the catalytic subunit of α-GCS (the rate-limiting enzyme in GSH biosynthesis). In addition, the catalytic subunit of DNA-PK is increased about twofold in some AA-resistant lines. This enzyme is involved in DNA double-strand break repair and has previously been found to be elevated in Adriamycin resistance. Our resistant cell lines were produced with chronic selection pressure. HD-SCR therapy may result in a qualitative difference in resistance by induction rather than genetic selection.

Other studies indicate a range of mechanisms for M resistance [30, 31, 32, 33, 34].

Studies of secondary neoplasms occurring after AA treatment, particularly in the adjuvant breast cancer setting, indicate that acute myelogenous leukemia and myelodysplasia occur 40 times more frequently after M compared CPA [35]. Generally, there is a high correlative and causal relationship between mutations and secondary cancer. This increased mutation rate could explain the increase in resistance associated with M [35]. We are analyzing these cells for mutator gene activation.

To our knowledge, this is the first report that the sequence of AAs may influence the therapeutic index. Because of the above, we have altered in our clinical trials the sequence from MT → CTC to CTC → MT. This study is ongoing and not yet sufficiently mature.

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