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Development and Cross-resistance Characteristics of a Subline of P388 Leukemia Resistant to 4′-(9-Acridinylamino)-methanesulfon-*m*-anisidide*

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Abstract—*A subline of P388 lymphocytic leukemia resistant to 4′-(9-acridinylamino)methanesulfon-*m*-anisidide* (m-*AMSA*) *was obtained by serial i.p. passage of tumor cells in mice treated i.p. with* m-*AMSA. Partial resistance was evident following eleven transplant generations. Complete resistance to* m-*AMSA* (<25% *increase in lifespan at a maximally tolerated dose) was evident following* 20 *transplant generations of drug exposure. Resistance to* m-*AMSA was a stable phenotype of P388/*m-*AMSA for at least* 42 *transplant generations following discontinuation of drug exposure. The growth kinetics and lethality of P388/*m-*AMSA in syngeneic mice were similar to those of the parental leukemia (P388/S). The pattern of cross-resistance of P388/*m-*AMSA was evaluated for a spectrum of antineoplastic agents with a variety of mechanisms of action. P388/*m-*AMSA was cross-resistant to* 4-(9-*acridinylamino)aniline derivatives substituted in either the acridine or aniline moieties. There was no cross-resistance to* N,N′-*diacridin-*9-*yl-*1,6-*hexanediamine. Among structurally unrelated drugs which bind to DNA, cross-resistance was evident to ellipticine, lucanthone, alkylamino-anthraquinones and some anthracyclines. P388/*m-*AMSA was quite sensitive to dactinomycin. Among the anthracyclines,* N-*dialkyl substituted compounds such as aclacinomycin A, cinerubin A, and* N-*diethyl-daunorubicin were active in P388/*m-*AMSA. Partial or complete cross-resistance was evident with most anthracyclines. P388/*m-*AMSA was sensitive to Vinca alkaloids, antimetabolites, alkylating agents and protein synthesis inhibitors. The subline was cross-resistant to the epipodophyllotoxins, VM-*26 *and VP*16-213.

INTRODUCTION

m-AMSA is a new agent which has shown useful activity in patients with acute myelogenous leukemia and has minimal activity in some solid tumors in early clinical trial [1–3]. *m*-AMSA binds to DNA [4], causes alkali-labile DNA single-strand breaks [5], and also reacts with sulfhydryl groups on small molecules (e.g., glutathione) and porteins [6]. The antineoplastic activity [7] and myelosuppressive effects [1] of *m*-AMSA are presumably due to DNA damage resulting from the intercalation of *m*-AMSA or a reactive metabolite [8]. Because of the interest in this new agent, we developed a resistant subline of P388 leukemia for the purpose of (a) providing a tool for the study of the biochemical pharmacology of *m*-AMSA, (b) for evaluation of *m*-AMSA analogs, and (c) for determination of the pattern of cross-resistance between *m*-AMSA and other antineoplastic agents. We had previously observed that a subline of P388 leukemia resistant to doxorubicin was completely cross-resistant to *m*-AMSA [9]. The development of an *m*-AMSA-resistant subline of P388 and its cross-resistance pattern to antineoplastic agents *in vivo* is presented herein.

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MATERIALS AND METHODS

The parental P388 leukemia (P388/S) was maintained by serial i.p. passage in syngeneic DBA/2 mice according to standard screening protocols [10]. The *m*-AMSA-resistant subline

was developed by serial i.p. passage of P388 cells in BALB/c × DBA/2 ($CD2F_1$) mice which were treated i.p. with *m*-AMSA at 4 mg/kg/day on Days 4–10. Cells were transplanted when ascites was evident or when tumor-bearing mice began to die. After nine transplant generations, the *m*-AMSA treatment schedule was changed so that mice received the drug on Days 1–7. After 24 transplant generations, a subline was passaged in untreated mice and evaluated for response to *m*-AMSA at 5, 13 and 42 transplant generations to assess stability of resistance in the absence of drug exposure. The cross-resistance studies reported herein were carried out between the 18th and 40th transplant generations of P388/*m*-AMSA using a subline which was continually passaged in *m*-AMSA-treated mice.

All drugs, including *m*-AMSA, which were used in these studies were obtained from the Drug Evaluation Branch, National Cancer Institute. For chemotherapy studies 10^6 cells of P388/S or P388/*m*-AMSA were inoculated i.p. in groups of eight $CD2F_1$ mice. In all experiments drugs were evaluated in P388/S and P388/*m*-AMSA simultaneously. Nine to eleven drugs plus *m*-AMSA were evaluated in each experiment. The drugs were tested at five dose levels, each dose level being 0.6 of the next highest. The dose levels were selected based on screening data so that the highest dose tested would be toxic. The treatment schedule used was intermittent treatment on Days 1, 5 and 9 for the majority of drugs. Other schedules were used when necessitated by schedule-dependency of a particular drug. Drugs were administered i.p. as solutions when possible or as suspensions in appropriate aqueous vehicles.

Each experiment included titrations of P388/S and P388/*m*-AMSA in untreated $CD2F_1$ mice (10^6–10^0 cells/mouse) so that differences in growth characteristics could be assessed and drug-induced cell kill could be compared in the two cell lines. Prolongation of median survival time relative to untreated controls (32 in each experiment) was determined.

Sensitivity or cross-resistance of P388/*m*-AMSA to a particular drug was assessed by comparing increase in lifespan and net change in tumor load at the end of therapy at the optimal dose of the drug with that obtained in P388/S, as described for other resistant cell lines by Schabel and co-workers [11, 12]. P388/*m*-AMSA was adjudged to be resistant to a drug if there was ≥3-log difference in the net change in tumor load at the end of treatment in P388/S or P388/*m*-AMSA. Partial cross-resistance was indicated by a 2 to 3-log difference in cells surviving therapy. No cross-resistance was assumed when there was less than a 2-log difference in the net change in tumor cell load following therapy of P388/S or P388/*m*-AMSA (or when cell kill of P388/*m*-AMSA was greater than that of P388/S). Cells surviving treatment were determined from the median survival following cessation of treatment (usually Day 9) at the optimal dose as determined from regression equations derived from the titration performed with each experiment. The equation used for calculation of net change in log tumor cell burden at the end of therapy is $(x - b/m) - y$, where x is the median survival time in days following cessation of therapy, b is the median time to death following inoculation of one cell of P388/S or P388/*m*-AMSA and m is the slope of the inoculum–survival time curve. These latter two factors were determined by regression analysis of titration data excluding long-term survivors. The value y is the tumor cell burden present at the start of therapy calculated as $6 + \log 2^{1/DT}$, where 6 is the original inoculum (10^6 cells) and DT, the cell population doubling time, is $-m \log 2$. We assumed that the tumor cell burden at death was about 6 g (6×10^9 cells) [11].

RESULTS

After 11 transplant generations of exposure to *m*-AMSA *in vivo*, the subline P388/*m*-AMSA was partially resistant to the drug (Fig. 1). A maximally tolerated dose of *m*-AMSA on an intermittent schedule (8 mg/kg, Days 1, 5 and 9) produced 112% ILS in mice bearing P388 and only 49% ILS in mice bearing P388/*m*-AMSA. The degree of resistance increased during subsequent transplantation in *m*-AMSA-treated mice; complete resistance (<25% ILS) to a maximally tolerated dose of *m*-AMSA was evident on the 20th and subsequent transplant generations. After 24 transplant generations, a subline was transplanted and maintained in mice without *m*-AMSA treatment. This subline was still completely resistant to *m*-AMSA after passage for 42 generations in untreated mice, indicating phenotypic stability of resistance to *m*-AMSA in this subline of P388 leukemia.

The growth kinetics of P388/S and P388/*m*-AMSA determined from parallel titrations in 9 experiments are shown in Fig. 2. The growth kinetics of the resistant line were virtually identical to those of the sensitive parental tumor line. The doubling times were 12.1 hr for P388/S and 11.6 hr for P388/*m*-AMSA, the TD_{50} (number of cells required to produce progressive disease in 50% of the mice) for both cell lines was one cell, the i.p. implantation of

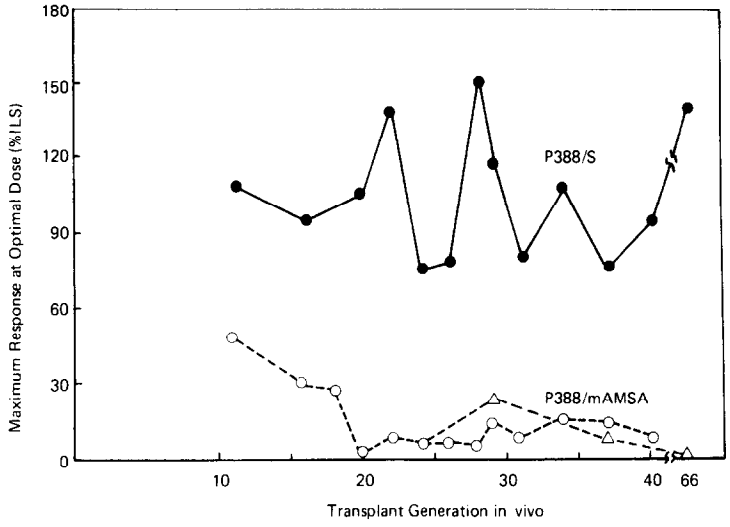


Fig. 1. Development and stability of resistance to m-*AMSA in a subline of P388 leukemia. The* m-*AMSA-resistant line was serially transplanted in mice which received* 4 mg/kg *of* m-*AMSA i.p. on Days* 4–10 *for* 9 *transplant generations and Days* 1–7 *on subsequent transplant generations* (O----O). *After* 24 *transplant generations a subline was serially transplanted without* m-*AMSA treatment* (Δ----Δ). *Each point represents the response to a maximally tolerated dose of* m-*AMSA administered i.p. on Days* 1, 5 *and* 9 *in groups of* 8 *mice bearing* 10^6 *parental P388/s cells* (●----●) *or the resistant subline.*

which resulted in leukemic death in about 21.5 days.

The resistance of P388/*m*-AMSA to a number of 9-aminoacridine antitumor agents is shown in Table 1. In 10 experiments, *m*-AMSA at its optimal dose increased lifespan of mice bearing P388/S by 82–167%. This corresponds to a net reduction in tumor cell burden at the end of treatment (Day 9). In P388/*m*-AMSA there was an increase in cell burden under treatment with a maximally tolerated dose of *m*-AMSA. P388/*m*-AMSA was cross-resistant to five 4′-(9-acridinylamino)aniline derivatives with various substitutions in the acridine and aniline moieties. The diacridine, *N,N′*-di-9-acridinyl-1,6-hexanediamine (NSC 219733), described by Canellakis *et al.* [13] was nearly as active against P388/*m*-AMSA as against P388/S.

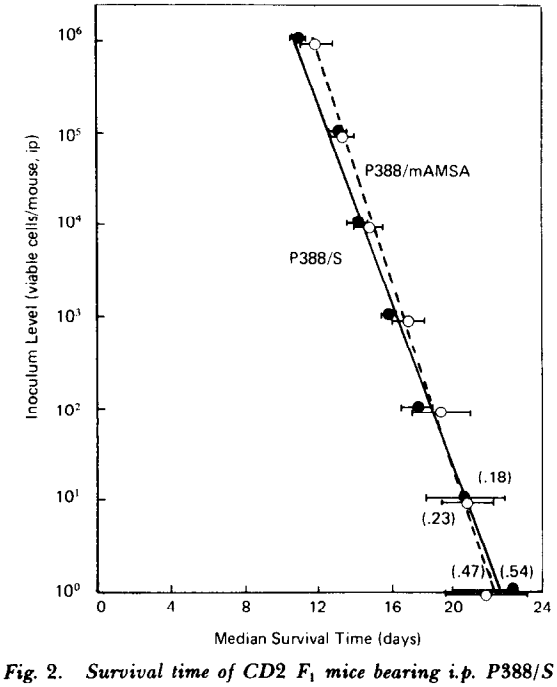


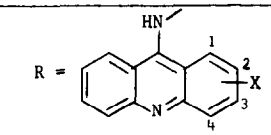
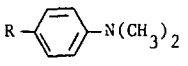
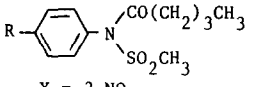
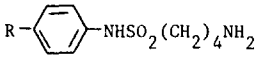
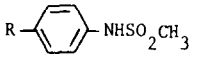
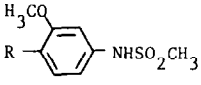
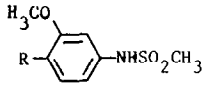
Fig. 2. Survival time of CD2 F_1 *mice bearing i.p. P388/S or P388/*m*-AMSA as a function of cell inoculum. Each point represents the mean ±S.D. of the median survival time at each inoculum level in* 9 *titrations* (8 *mice per group in each of the* 9 *titrations). Values in parentheses at inoculum levels of* 10^1 *and* 10^0 *are proportion of mice which survived for* 45 *days without evidence of tumor.*

A spectrum of semisynthetic and naturally occurring anthracycline antibiotics was evaluated for activity in P388/*m*-AMSA (Table 2). Cross-resistance was clearly evident to a number of these compounds, including doxorubicin and daunorubicin. However, some anthracyclines, notably the trisaccharides, cinerubin A and aclacinomycin A, and *N*-dialkyl derivatives of daunorubicin were active in P388/*m*-AMSA.

Cross-resistance was also evident in P388/*m*-AMSA for a number of other DNA-binding agents (Table 3), including ellipticine, lucanthone, the alkylaminoanthraquinones, mitoxantrone and ametantrone [14] and the anthracenedicarboxaldehyde derivative, bisantrene [15]. On the other hand, dactinomycin and camptothecin had equivalent activity in the parental and *m*-AMSA-resistant sublines of P388 leukemia.

Four antitumor plant products which act by virtue of binding to tubulin, namely, vinblastine, vincristine, taxol and maytansine, showed no evidence of cross-resistance in P388/*m*-AMSA (Table 4). Similarly, P388/*m*-

Table 1. Cross-resistance of P388/m-AMSA to 9-aminoacridine antitumor agents

NSC No.	R = 	Expt No.	%ILS at Optimal Dose* P388/S	%ILS at Optimal Dose* P388/m-AMSA	Δ Log Cells Post-Rx† P388/S	Δ Log Cells Post-Rx† P388/m-AMSA	S or R§
13002		2	82	4	-0.4	+3.0	R
143106	 X = 3-NO_2	4	82	0	-0.4	+3.0	R
143107	 X = 3-NO_2	4	95	0	-1.5	+3.0	R
156304	 X = 3-CH_3 ; 4-CH_3	4	64	0	+0.6	+3.0	PR
219733	R-$(CH_2)_6$-R	2	68	38	+0.4	+2.1	S
		8	85	50	+0.8	+1.7	
235752	 X = 3-CF_3 ; 2-NH_2	6	124	9	-3.4	+3.0	R
249992	 (m-AMSA)	1	108	27	-3.5	+3.0	R
		2	127	0	-3.4	+3.0	
		3	140	14	-3.1	+3.0	
		4	82	0	-0.6	+3.0	
		5	96	4	-2.5	+3.0	
		6	167(1/8)	13	-6.8	+3.0	
		7	86	18	-0.9	+3.0	
		8	125	9	-2.6	+3.0	
		9	95	14	-1.6	+3.0	
		10	95	18	-3.9	+1.8	

*Greatest increase in lifespan relative to untreated controls among five dose levels administered i.p. on Days 1, 5 and 9 for all drugs except NSC 219733, which was administered i.p. on Days 1–9. Fractions in parentheses are long-term tumor-free survivors on Day 45. *m*-AMSA was given i.p. on Days 1 and 5 in Experiment 10.

†Change in tumor cell burden in logs at the end of therapy on Day 9 calculated as described in Materials and Methods.

§Sensitive (S), partially resistant (PR) or resistant (R) according to criteria described in Materials and Methods.

AMSA retained sensitivity to the natural product protein synthesis inhibitors emetine, anguidine and homoharringtonine. As shown in Table 5, P388/*m*-AMSA was fully sensitive to antimetabolites—methotrexate, 5-fluorouracil, 5-azacytidine, and arabinosylcytosine—and to alkylating agents—melphalan, cyclophosphamide, mitomycin C, *cis*-platinum and BCNU. The alkylating agents were somewhat more active as a class against P388/*m*-AMSA than against P388/S.

Among the other agents evaluated against

Table 2. Cross-resistance of P388/m-AMSA to anthracyclines

NSC No.	Drug*	Expt No.	%ILS at Optimal Dose†		Δ Log cells post-Rx†		S or R†
			P388/S	P388/m-AMSA	P388/S	P388/m-AMSA	
18334	Cinerubin A	6	67	117	+1.2	-3.5	S
		8	55	55	+2.2	+1.4	
82151	Daunorubicin	2	68	25	+0.4	+3.0	PR
		4	59	23	+0.9	+3.0	
		7	59	32	+0.5	+3.0	
123127	Doxorubicin	1	142(1/8)	50	-5.8	+0.9	R
		2	127(2/8)	25	-3.4	+3.0	
		10	105(3/8)	36	-4.6	+0.3	
149584	DOX-14-octanoate	7	100	36	-1.6	+3.0	R
164011	Rubidazone	8	80	27	+1.0	+3.0	PR
180024	Carminomycin	7	59	27	+0.5	+3.0	PR
208734	Aclacinomycin A	6	95	126	-1.1	-0.8	S
		8	80	50	+1.0	+1.7	
246131	AD32	6	110(1/8)	22	-2.2	+3.0	R
254681	5-IminoDAU	7	59	0	+0.5	+3.0	PR
256438	4-DemethoxyDOX	7	136(1/8)	27	-3.5	+3.0	R
256439	4-DemethoxyDAU	7	68	27	0	+3.0	R
258812	N-DimethylDAU	8	105	68	-0.8	+0.6	S
265205	N-DiethylDAU	8	70	64	+1.8	+0.8	S
265450	Nogamycin	7	55	45	+0.8	+2.4	S
268242	N-BenzylDAU	8	110	18	-1.3	+3.0	R
269148	7-con-O-Methyl-nogarol	6	148	13	-5.3	+3.0	R
269433	N-BenzylDOX	8	105	41	-0.9	+2.4	R

*All drugs were administered i.p. on Days 1, 5 and 9, except doxorubicin, in Experiment 10, was given on Days 1 and 5.
†See footnotes to Table 1.

P388/*m*-AMSA (Table 6), cross-resistance was clearly evident to the epipodophyllotoxins, VM-26 and VP16-213. The resistant line retained sensitivity to a terephthalanilide derivative (NSC 57153), a related quinolinium derivative (NSC 176319), razoxane (ICRF-159) and neocarzinostatin.

DISCUSSION

The cross-resistance pattern of the *m*-AMSA-resistant subline of P388 leukemia described in this report is relatively discrete. P388/*m*-AMSA is cross-resistant to many, but not all, agents which bind to DNA by intercalation. The only agents other than known DNA intercalators to which P388/*m*-AMSA proved cross-resistant are the epipodophyllotoxin glucosides, VP16-213 and VM-26. Although these agents are structurally related to the mitotic spindle poisons, podophyllotoxin and peltatin, mechanistic studies indicate that VP16-213 and VM-26 are not mitotic spindle poisons; they do not bind to tubulin [16], nor do they inhibit microtubule assembly [17] or arrest cells in mitosis [18]. VP16-213 and/or VM-26 have been shown to (a) produce cell cycle progression blockade in S and G_2 phases rather than in M [19], (b) inhibit thymidine and uridine incorporation into nucleic acids to a greater extent than could be due to inhibition of nucleoside uptake (an effect these compounds have in common with known mitotic spindle poisons) [17], and (c)

Table 3. Cross-resistance of P388/m-AMSA to nonanthracycline DNA-binding agents

NSC No.	Drug*	Expt No.	%ILS at optimal dose† P388/S	%ILS at optimal dose† P388/m-AMSA	Δ log cells post-Rx† P388/S	Δ log cells post-Rx† P388/m-AMSA	S or R†
3053	Dactinomycin	1	75	115(1/8)	-1.4	-4.4	S
		5	117(2/8)	117(1/8)	-4.6	-3.9	
14574	Lucanthone	2	73	0	-2.2	+3.0	R
		5	38	0	-0.8	+3.0	
71795	Ellipticine	1	104	8	-3.2	+3.0	R
		2	77	0	-0.2	+3.0	
		9	95	9	-1.6	+3.0	
		10	68	0	-2.1	+3.0	
100880	Camptothecin	1	100	88	-3.0	-2.2	S
279836	Mitoxantrone	2	>200(6/8)	50	-6.6	+1.0	R
		4	>200(5/8)	35	-6.6	+2.6	
		9	155(2/8)	64	-4.6	+0.5	
		10	123(2/8)	55	-5.8	-1.1	
287513	Ametantrone	4	86	12	-0.9	+3.0	R
337766	Bisantrene	10	100(1/8)	27	-4.2	+0.9	R

*All drugs were administered i.p. on Days 1, 5 and 9, with the exception of lucanthone which was given i.p. on Days 1–5, and all drugs in Experiment 10 in which treatment was i.p. on Days 1 and 5.
†See footnotes to Table 1.

Table 4. Activity in P388/m-AMSA of tubulin binders and protein synthesis inhibitors

NSC No.	Drug*	Expt No.	%ILS at optimal dose† P388/S	%ILS at optimal dose† P388/m-AMSA	Δ log cells post-Rx† P388/S	Δ log cells post-Rx† P388/m-AMSA	S or R†
33669	Emetine	1	62	50	-0.6	+0.9	S
49842	Vinblastine	1	79	69	-1.6	-0.7	S
67574	Vincristine	1	108	96(1/8)	-3.5	-2.8	S
125973	Taxol	5	50	29	+1.0	+2.4	S
141537	Anguidine	3	70	64	+1.1	+2.5	S
141633	Homoharringtonine	3	65	68	+1.4	+0.6	S
153858	Maytansine	2	91	83	-1.0	-1.9	S

*All drugs were administered i.p. on Days 1, 5 and 9.
†See footnotes to Table 1.

Table 5. Activity in P388/m-AMSA of antimetabolites and alkylating agents

NSC No.	Drug*	Expt No.	%ILS at optimal dose†		Δ log cells post-Rx†		S or R†
			P388/S	P388/m-AMSA	P388/S	P388/m-AMSA	
740	Methotrexate	1	117	92	-4.0	-2.5	S
8806	Melphalan	3	185(1/8)	>200(8/8)	-5.8	-6.6	S
		10	118(1/8)	>200(5/8)	-5.4	-6.7	
19893	5-Fluorouracil	1	67	65	-0.8	-0.4	S
26271	Cyclophosphamide	1	>200(3/8)	>200(6/8)	-6.5	-6.6	S
		10	118	>200(6/8)	-5.4	-6.7	
29680	Mitomycin C	3	130(1/8)	141(3/8)	-2.5	-3.7	S
63878	Arabinosylcytosine	6	181	165	-6.8	-6.6	S
102816	5-Azacytidine	3	190	182	-6.1	-6.5	S
119875	Cisplatin	3	180(2/8)	>200(5/8)	-5.5	-6.6	S
409962	BCNU	6	>200(3/8)	>200(8/8)	-6.8	-6.6	S

*All drugs were administered i.p. on Days 1, 5 and 9, except in Experiment 10, in which treatment was i.p. on Days 1 and 5.
†See footnotes to Table 1.

Table 6. Activity in P388/m-AMSA of miscellaneous antitumor agents

NSC No.	Drug*	Expt No.	%ILS at optimal dose†		Δ log cells post-Rx†		S or R†
			P388/S	P388/m-AMSA	P388/X	P388/m-AMSA	
57153	Terephthalanilide	3	105	91	-1.0	-0.3	S
122819	VM-26 (Teniposide)	5	>200(7/8)	46	-6.7	+1.2	R
129943	Razoxane (ICRF-159)	3	115	100	-1.6	-1.0	S
141540	VP16-213 (Etoposide)	3	>200(3/8)	73	-6.6	+0.9	R
		4	>200(1/8)	58	-6.6	+0.1	
157365	Neocarzinostatin	4	73	50	0	+1.0	S
176319	Cain's Quinolinium	3	110	82	-1.3	+0.3	S

*All drugs were administered i.p. on Days 1, 5 and 9.
†See footnotes to Table 1.

produce DNA single-strand breaks in cultured cells [20]. These properties are similar to those of known DNA intercalating agents such as doxorubicin [21–23], mitoxantrone [14, 24, 25] and *m*-AMSA [5, 26]. Thus, it appears feasible that VP16-213 and VM-26 act as DNA binders.

A doxorubicin-resistant subline of P388 leukemia described earlier [9] and P388/*m*-AMSA are mutually cross-resistant to doxorubicin and *m*-AMSA. However, the patterns of cross-resistance of these two sublines are quite different. P388/DOX is cross-resistant to many drugs which are active against P388/*m*-AMSA including the spindle poisons and protein synthesis inhibitors, as well as dactinomycin, razoxane, terephthalanilide derivatives, Cain's quinolinium and anthracyclines such as cinerubin A, aclacinomycin A and *N*-dimethyldaunorubicin. Cinerubin A and aclacinomycin A contain the basic sugar rhodosamine which is *N*-dimethyldaunosamine. It would appear that dialkylation of the sugar amino group is important in determining activity of anthracycline derivatives in P388/*m*-AMSA. However, monoalkylation as in *N*-benzyldaunorubicin or acylation as in AD-32 does not give activity in P388/*m*-AMSA. The mechanism of resistance of P388/DOX to several agents (i.e., doxorubicin,

dactinomycin, and emetine) is enhanced active efflux [27–30]. Our studies (D. Kessel and R. K. Johnson, unpublished observations) indicate that cellular uptake and efflux of daunorubicin and *m*-AMSA are not modified in P388/*m*-AMSA, though resistance to these agents is evident. However, binding of *m*-AMSA and daunorubicin to the nuclear fraction appears to be impaired in P388/*m*-AMSA.

Further study of the mechanism of resistance of P388/*m*-AMSA could be useful in gaining a greater understanding of the cytotoxic mechanism of DNA intercalating agents and in differentiating between structurally related but mechanistically different agents such as doxorubicin and aclacinomycin A.

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