Cancer Chemother Pharmacol (1990) 26: 250–256

Cancer Chemotherapy and Pharmacology

Patterns of cross-resistance in a multidrug-resistant small-cell lung carcinoma cell line*

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Received 30 November 1989/Accepted 15 February 1990

Summary. H69AR is a multidrug-resistant human small-cell lung carcinoma cell line that was selected in doxorubicin and has previously been shown to be cross-resistant to a variety of natural-product-type anticancer drugs. H69AR is unlike many other multidrug-resistant cell lines in that it does not overexpress P-glycoprotein. In the present study, the drug sensitivity and cross-resistance patterns of H69AR cells were further characterized. A total of 15 drugs belonging to a number of chemical classes were screened. These compounds included anthracyclines, DNA binders (anthrapyrazoles, benzothiopyranoindazoles, and pyrazoloacridines), and lipophilic antifolates. The alkylating agent melphalan and the antimetabolite cytosine arabinofuranoside (Ara-C) were also tested. In general, the drug sensitivity and cross-resistance profiles of H69AR cells were consistent with those reported by others using other drug-resistant cell lines. However, there were several unexpected instances of cross-resistance. Thus, the H69AR cell line was more resistant than its parent cell line to the potent 3'-deamino-3'-(3-cyano-4-morpholinyl) doxorubicin, bisantrene, the pyrazoloacridine PD 114541, Ara-C, and melphalan. In addition, no cross-resistance to the four lipophilic antifolates tested, including trimetrexate, was found. The absence of a consistent pattern among the various drug-resistant cell lines indicates that assumptions about the efficacy of anticancer drugs in multidrug resistance should be made with caution.

Introduction

The development of multidrug resistance (MDR) presents a major obstacle to the successful treatment of many human tumours. P-glycoprotein, a plasma membrane efflux pump, has been shown to be essential for the expression of the MDR phenotype in many in vitro model systems [4, 12] and appears to be clinically relevant in some malignancies [11, 13, 23, 27, 32]. However, there are some tumour types, such as lung cancers [26], in which P-glycoprotein does not appear to play a clinical role. In addition, there are a number of MDR cell lines that do not overexpress P-glycoprotein, and other mechanisms (e.g., altered topoisomerase II activity, altered intracellular drug distribution, and changes in drug detoxification pathways) have been implicated [7, 20, 28, 29, 37, 42]. One such cell line is the small-cell lung carcinoma cell line, H69AR, derived in this laboratory [29].

Two pharmacological approaches are being followed in efforts to overcome the problem of MDR. One involves the use of agents that enhance the chemosensitivity of known antineoplastic agents [14]. The other approach involves the screening of analogues of drugs in current clinical use as well as novel compounds against resistant cell lines. Numerous in vitro studies of both types have been reported using cell lines that overexpress P-glycoprotein [2, 16, 19, 25, 36, 44, 45] (and others). However, there is limited data with respect to cell lines in which resistance is not mediated by P-glycoprotein [6]. In this paper, the patterns of cross-resistance and drug sensitivity of the non-P-glycoprotein-mediated MDR small-cell lung carcinoma cell line, H69AR [29], are described. This information may be useful in the development of more effective therapeutic strategies in lung cancer and other tumours in which P-glycoprotein is not believed to play a major role in resistance.

Materials and methods

Drugs and chemicals. Doxorubicin, 4-demethoxydaunorubicin (idarubicin), and 4'-deoxy-4'-iododoxorubicin were provided by Adria Laboratories (Columbus, Ohio), and 3'-deamino-3'-(3-cyano-4-morpholinyl)doxorobucin (MRA-CN) was provided by Dr. E. M. Acton (NIH, Bethesda, Md.). Bisantrene was a gift of American Cyanamid Co. (Pearl, N. Y.). The anthrapyrazoles [piroxantrone (CI-942), CI-937, and CI-941), the pyrazoloacridines PD 115934 and PD 114541, and the benzothiopyranoindazoles PD 114595 and CI-958 (PD 118484) were

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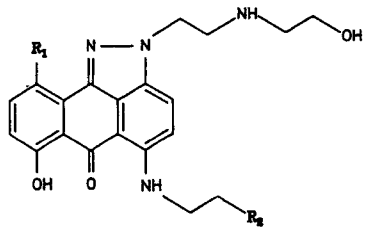
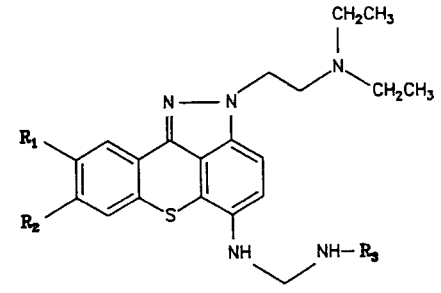
(A)



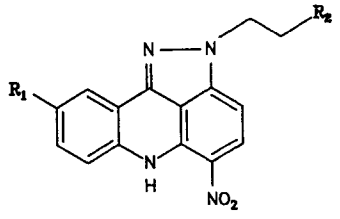
	R_1	R_2
CI-942	OH	CH_2NH_2
CI-937	OH	$NHCH_3$
CI-941	H	$NHCH_2CH_2OH$

(B)



	R_1	R_2	R_3
PD118,484	H	OH	H
PD114,595	OH	H	CH_2CH_2OH

(C)

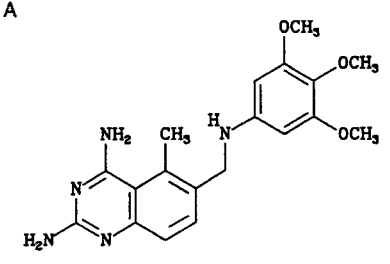


	R_1	R_2
PD115,934	OCH_3	CH_2NMe_2
PD114,541	OH	NEt_2

Fig. 1 A–C. Molecular structures of A anthrapyrazoles, B benzothiopyranoindazoles, and C pyrazoloacridines

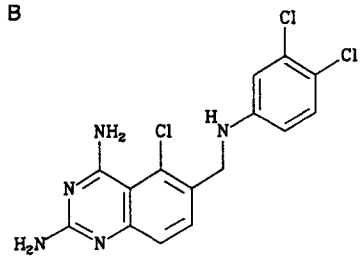
A

5:D-Gluco



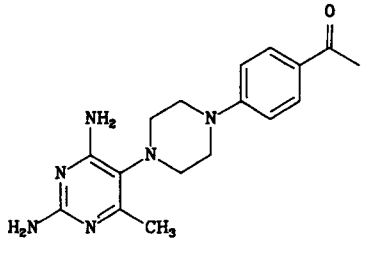
PD 074013-0714 Trimetrexate

B



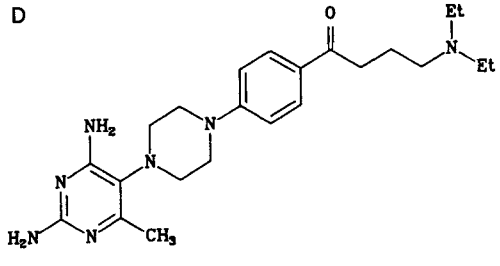
PD 075197

C



PD 115748

D



PD 131907

Fig. 2 A–D. Molecular structures of the antifolates A trimetrexate, B PD 75197, C PD 115748, and D PD 131907

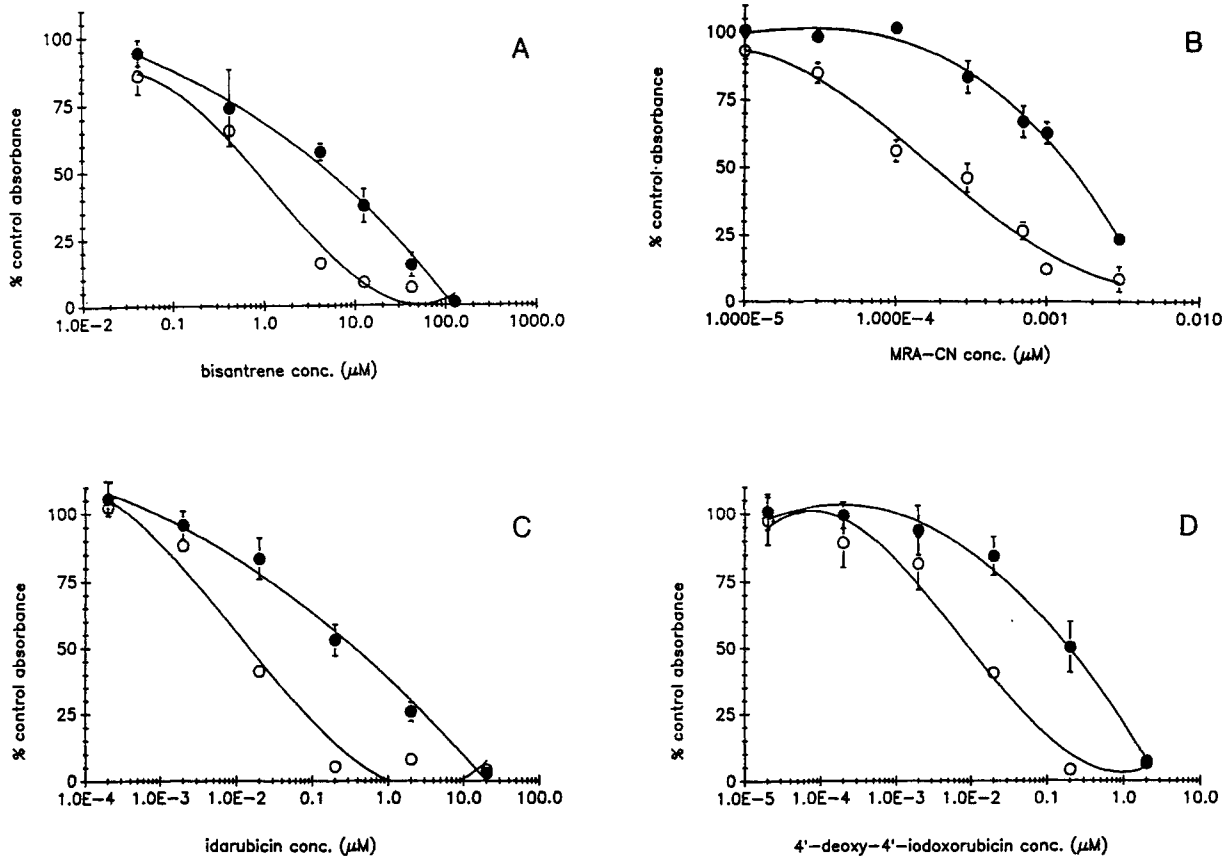


Fig. 3 A–D. Effect of anthracyclines and related compounds on the growth of H69 cells (○) and multidrug-resistant H69AR cells (●), as measured by the MTT assay. The compounds tested included **A** bisantrene, **B** MRA-CN, **C** idarubicin, and **D** 4'-deoxy-4'-iododoxorubicin. Each point represents the mean ± SD of four determinations in a typical experiment

Table 1. Cross-resistance of H69AR cells to anthracycline-type compounds

Drug	IC_{50}(n*M*)[a]:		
	H69	H69AR	Resistance[b] (*n*-fold)
Doxorubicin	74 ± 45 (*n* = 28)	4,002 ± 2,310[c] (*n* = 16)	54
Bisantrene	675 ± 350 (*n* = 4)	2,901 ± 1,250[c] (*n* = 3)	4
MRA-CN	0.157 ± 0.061 (*n* = 3)	0.853 ± 0.395[c] (*n* = 3)	6
Idarubicin	25.6 ± 10.8 (*n* = 3)	290[c] (*n* = 2)	11
4'-Deoxy-4'-iododoxorubicin	18.0 ± 6.1 (*n* = 3)	385[c] (*n* = 2)	21

[a] Values given represent the mean ± SD of the results obtained in two or more independent experiments

[b] IC_{50} H69AR: IC_{50} H69

[c] Significantly different from H69 values ($P < 0.05$) according to the unpaired *t*-test

provided by Dr. H. Showalter (Parke-Davis, Ann Arbor, Mich.). The molecular structures of these compounds are shown in Fig. 1. The quinazoline antifolates trimetrexate (PD 74013-714) and PD 75197 and the diaminopyrimidines PD 115748 and PD 131907 were provided by Dr. W. D. Klohs (Parke-Davis). The molecular structures of these compounds are shown in Fig. 2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cytosine arabinofuranoside (Ara-C), and melphalan were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cytotoxicity testing. The H69 cell line was provided by J. Minna (NIH, Bethesda, Md.) and its MDR variant, H69AR, has been described previously [29]. These cell lines were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) (Flow Laboratories) and 4 m*M* L-glutamine at 37° C in a 95% air/5% CO_2 atmosphere and were free of mycoplasma contamination. Cytotoxicity was measured using a modification of the MTT assay, which has previously been described [5, 30]. In this assay, the tetrazolium salt MTT is converted to a blue formazan product by dehydrogenase enzymes that are active only in living cells. H69 and H69AR cells were seeded into microtitre plates at 2.5×10^4 cells/well in a volume of 100 μl. Drugs were added 24 h later in a 100-μl volume at twice the desired final concentration. Stock solutions of idarubicin, doxorubicin, MRA-CN,4'-deoxy-4'-iododoxorubicin, Ara-C, PD 114595, PD 114541, trimetrexate, piroxantrone, CI-937, CI-941, CI-958, and PD 115934 were prepared in tissue-culture medium and diluted as required. PD 75197, PD 115748, and PD 131907 were dissolved in dimethyl sulfoxide and diluted in RPMI 1640/5% FBS as required. Melphalan was dissolved in acidified isopropanol and bisantrene, in acidified medium prior to dilution in RPMI 1640/5% FBS. After a 4-day exposure to drug, 100 μl medium was removed, 25 μl MTT (2 mg/ml PBS) was added, and the plates were incubated for 3 h at 37° C. The resulting formazan crystals were solubilized by the addition of 100 μl isopropanol: 1 *N* HCl (24:1), thorough mixing, and further in-

Table 2. Cross-resistance of H69AR cells to anthrapyrazoles, benzothiopyranoindazoles and pyrazoloacridines

Compound[a]	Experiment	IC_{50} (μM)[b]:		Resistance[c] (*n*-fold)
		H69	H69AR	
Anthrapyrazoles:				
CI-942	1	5.9	>10	>1.7
(piroxantrone)	2	1.9	>10	>5.3
CI-937	1	0.20	2.1	11
	2	0.22	2.4	11
CI-941	1	0.050	0.55	11
	2	0.065	0.81	13
Benzothiopyranoindazoles:				
PD 114595		0.042 ± 0.013 ($n = 3$)	1.068 ± 0.623[d] ($n = 4$)	25
CI-958 (PD 118484)		0.44 ± 0.19 ($n = 3$)	1.22 ± 0.30[d] ($n = 3$)	2.8
Pyrazoloacridines:				
PD 115934		0.35 ± 0.07 ($n = 3$)	0.91 ± 0.09[e] ($n = 3$)	2.6
PD 114541		0.028 ± 0.006 ($n = 3$)	6.13 ± 3.30[d] ($n = 3$)	219

[a] Molecular structures of these compounds are shown in Fig. 1

[b] Where fewer than three experiments were done or it was not possible to calculate an IC_{50} because the percentage of control absorbance did not fall below 50% over the range of concentrations tested, the results of individual experiments are given. Otherwise, the results shown represent the mean ± SD of results obtained in three or more independent experiments

[c] IC_{50} H69AR : IC_{50}H69

[d] Significantly different from H69 valaues ($P < 0.05$) according to the unpaired *t*-test

[e] Significantly different from H69 values ($P < 0.01$) according to the unpaired *t*-test

cubation for 1 h at 37° C. The plates were scanned at 570 nm with a Dynatech MR600 microtitre plate reader. Within each experiment, determinations were done in quadruplicate and each drug was tested in two or more separate experiments. In addition, a doxorubicin dose-response curve was included as an internal control for each day's experiments. Controls consisted of wells with no cells and wells with cells plus vehicle (baseline absorbance). Results were expressed as a percentage of the baseline absorbance at 570 nm and the IC_{50} was defined as being the dose of drug that reduced this absorbance to 50% of control values. Statistical analysis was done using an unpaired *t*-test, with a significance level of 0.05.

Results

The cytotoxic effects of four anthracycline-type compounds were determined in the first series of experiments. Typical dose-response curves for bisantrene, MRA-CN, idarubicin, and 4'-deoxy-4'-iododoxorubicin are shown in Fig. 3; the IC_{50}s for these compounds are summarized in Table 1. H69AR cells exhibited a significant cross-resistance to all four compounds. MRA-CN had the highest activity, and cytotoxicity to both the H69 and H69AR cell lines was observed at concentrations of <1 n*M*. The activities of the two anthracyclines idarubicin and 4'-deoxy-4'-iododoxorubicin were similar and moderately greater than that of doxorubicin. Bisantrene was less toxic than doxorubicin to H69 cells but the two drugs showed similar toxicity to H69AR cells.

In the second series of experiments, the cytotoxic effects of seven DNA-binding compounds were tested and the results are summarized in Table 2. Where IC_{50} values were obtained, the relative resistance of H69AR cells to these agents ranged from 2- to 219-fold. The three anthrapyrazoles varied considerably in their toxicity to H69 cells: piroxantrone (CI-942) was the least active, CI-937 showed intermediate cytotoxicity, and CI-941 was the most toxic. The two benzothiopyranoindazoles displayed about a 10-fold difference in their activity against H69 cells but showed similar toxicity to H69AR cells. This resulted in a modest cross-resistance of H69AR cells to CI-958 (2.8-fold) as compared with PD 114595 (25-fold). The two pyrazoloacridines also varied about 10-fold in their activity against H69 cells, whereas the relative cytotoxicity of these two compounds to H69AR cells was reversed. These differences resulted in a large relative resistance of 219-fold for PD 114541 and a modest relative resistance of 2.6-fold for PD 115934.

In the third group of experiments, four antifolates (Fig. 2) were evaluated for their cytotoxic effects; the results of these experiments are presented in Table 3. Only one of the four compounds, the diaminopyridine PD 115748, exhibited significant differential cytotoxicity to H69 and H69AR cells. In this case, however, the H69 cells were more resistant to PD 115748 than were H69AR cells, giving a ratio of IC_{50} H69AR: IC_{50} H69 amounting to 0.3. It should be noted that there was considerable difficulty in getting this compound completely into solution at a concentration of dimethylsulfoxide that did not itself cause toxicity.

In the last series of experiments, melphalan and Ara-C were tested; typical dose-response curves are shown in Fig. 4. The IC_{50} of Ara-C on H69 cells was 0.589 ± 0.442 μ*M* ($n = 4$). The dose-response curve for Ara-C on H69AR cells was very flat (Fig. 4 A), and in most experiments the IC_{50} was not achieved by the maximal concentration of drug tested (0.5 m*M*). Thus, it was not

Table 3. Cross-resistance of H69AR cells to trimetrexate and other antifolate compounds

Drug[b]	IC_{50} (μM)[a]:		
	H69	H69AR	Ratio[c]
Trimetrexate (PD 074013-0714)	0.025 ± 0.012	0.039 ± 0.027[d]	1.6
PD 075197	0.068 ± 0.062	0.065 ± 0.114[d]	1.0
PD 115748	1.244 ± 0.425	0.334 ± 0.394[e]	0.3
PD 131907	0.376 ± 0.376	0.139 ± 0.176[d]	0.4

[a] Values given represent the mean ± SD of results obtained in three or more independent experiments
[b] Molecular structures are given in Fig. 2
[c] IC_{50} H69AR : IC_{50} H69
[d] Not significantly different from H69 values ($P > 0.1$)
[e] Significantly different from H69 values ($P < 0.05$)

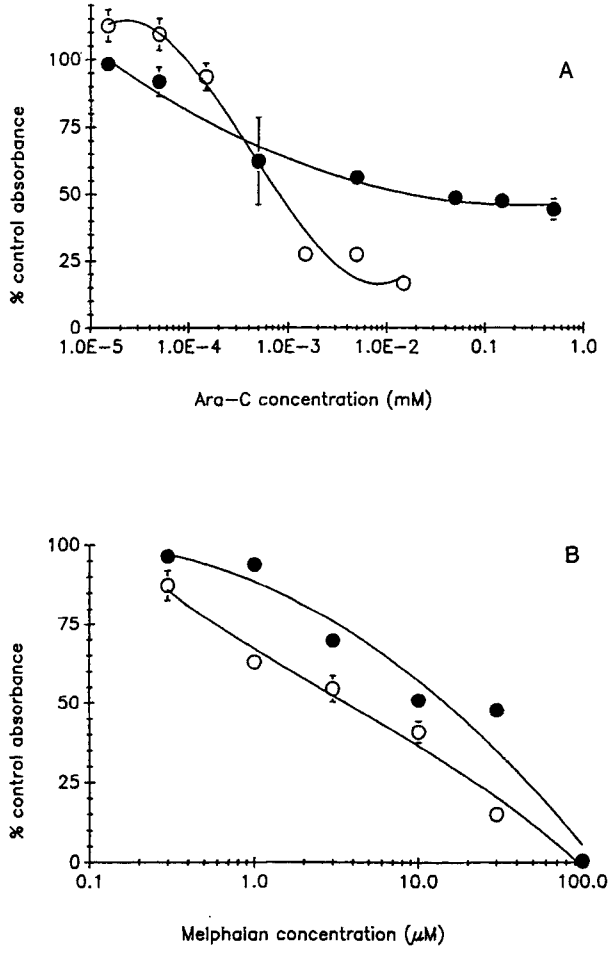


Fig. 4 A, B. Effect of **A** Ara-C and **B** melphalan on the growth of H69 cells (○) and multidrug-resistant H69AR cells (●), as measured by the MTT assay. Each point represents the mean ± SD of quadruplicate determinations in a typical experiment

possible to determine accurately the extent of cross-resistance to Ara-C. H69AR cells displayed a 2-fold resistance to melphalan (IC_{50} H69AR, 14.54 ± 5.99 μM, vs IC_{50} H69, 7.28 ± 3.08 μM ($n = 5$) that was statistically significant ($P < 0.05$) (Fig. 4B).

Discussion

The present study was undertaken to evaluate the activity of a panel of antineoplastic agents belonging to a number of different chemical classes against the H69AR cell line. This MDR cell line was selected in doxorubicin and does not overexpress P-glycoprotein. Nevertheless, it is cross-resistant to a variety of natural products or their semi-synthetic derivatives, including daunomycin, epirubicin, menogaril, and mitoxantrone, as well as to acivicin, etoposide, gramicidin D, colchicine, and the *Vinca* alkaloids vincristine and vinblastine [29].

The use of doxorubicin in the practice of clinical oncology is often restricted by a cumulative dose-limiting cardiotoxicity as well as by the development of resistance. These limitations have provided the stimulus for research efforts that have resulted in the synthesis of a vast number of anthracycline analogues. Cell lines that overexpress P-glycoprotein have been found to display cross-resistance to most of these analogues, although in many cases this resistance is somewhat diminished as compared with that to doxorubicin [8, 19, 21, 33, 45]. Nevertheless, there are some analogues, most notably, 9-alkyl [8, 19, 34, 45] and morpholinyl derivatives such as MRA-CN [1, 43], which have retained their activity against resistant cell lines to a large degree. As in previous reports with other cell lines [37, 40], MRA-CN was found to be considerably more active than doxorubicin against the H69 and H69AR cell lines (Table 1). However, in contrast to the findings of other investigators, who observed no (or minimal) cross-resistance to MRA-CN [37, 40, 43], the H69AR cells still displayed a 6-fold resistance to this compound (Fig. 3B, Table 1). 4'-Deoxy-4'-iododoxorubicin and idarubicin have shown reduced cross-resistance in other model systems [3, 8, 9, 19, 33], and similar results were found with H69 and H69AR cells (Table 1). Thus, the three anthracyclines tested in the present study, including the highly active MRA-CN, all showed differential toxicity to the sensitive and resistant small-cell lung carcinoma cell lines, albeit to a lesser degree than doxorubicin (Fig. 3C, D; Table 1). A mitoxantrone-selected, drug-resistant colon carcinoma cell line that does not overexpress P-glycoprotein has been found to be cross-resistant to doxorubicin but not to vinblastine or bisantrene [10]. By contrast, H69AR cells are cross-resistant to both the *Vinca* alkaloids [29] and bisantrene (Table 1, Fig. 3A).

The anthrapyrazoles (Fig. 1A) are modified anthracenediones that were developed in an effort to identify DNA-binding antitumour agents with a lower potential for cardiotoxicity than the anthracyclines [18, 38, 39]. In agreement with our findings (Table 2), Klohs et al. [25] observed that piroxantrone was considerably less cytotoxic than doxorubicin to murine P388 cells. These investigators also found that the P388R cell line, which overexpresses P-glycoprotein, displayed cross-resistance to piroxantrone and CI-937, as does the H69AR cell line (Table 2). H69AR cells were cross-resistant to the anthrapyrazole CI-941, as has been reported for other MDR cell lines [8]. The related benzothiopyranoindazoles (Fig. 1B) have been tested in two rodent MDR cell lines (Dr. W. Klohs, personal communication). Cross-resistance to both CI-958 (PD 118484)

and PD 114595 was observed in one cell line, whereas in the other, cross-resistance was seen only with CI-958. In all cases, the level of resistance was moderate ($\leq$5-fold). With respect to H69AR cells, the cross-resistance to CI-958 was similar in the rodent cell lines but resistance to PD 114595 was much higher (25-fold); the structural basis for this difference in activity of the two benzothiopyranoindazoles is unknown. The pyrazoloacridines (Fig. 1C) have been reported to have selective activity against cell lines derived from solid tumours in vitro [35]. In a study using three MDR cell lines, Sebolt et al. [36] found no cross-resistance to PD 115934 in vitro; similarly, the cross-resistance of H69AR cells to PD 115934 was slight (2.6-fold) (Table 2). In contrast, H69AR cells displayed considerable cross-resistance to PD 114541 (219-fold), as compared with the low level of cross-resistance (2- to 3-fold) observed in the Sebolt et al. study [36]; the structural basis for this difference in activity against H69AR cells shown by the two pyrazoloacridines is also unknown.

Analogues to the antifolate methotrexate have been developed in an attempt to overcome the resistance that frequently occurs after exposure to this drug. One approach has been to synthesize analogues of greater lipophilicity than the parent compound, which can be expected to get into the cell by diffusion rather than by the methotrexate transport system [41]. Although these agents have been effective in circumventing methotrexate resistance [22], cross-resistance to trimetrexate (Fig. 2A) and some other lipophilic antifolates has been observed in several MDR cell lines that overexpress P-glycoprotein [2, 24, 31]. In contrast, significant cross-resistance to trimetrexate was not observed in H69AR cells (Table 3). The other quinazoline antifolate tested, PD 75197 (Fig. 2B), was also equally toxic to H69 and H69AR cells. It is noteworthy that cross-resistance to PD 75197 was not observed in the murine MDR cell line that did exhibit cross-resistance to trimetrexate (Dr. W. Klohs, personal communication). The two diaminopyrimidine antifolates PD 115748 and PD 131907 (Fig. 2C, D), were generally less cytotoxic than the quinazolines, and no cross-resistance in H69AR cells was observed; in contrast, a collateral sensitivity to these two agents was noted, although this effect was significant only in the case of PD 115748 (Table 3). These findings contrast with those reported in a P388 MDR cell line in which significant cross-resistance (111-fold) was observed with PD 131907 but not PD 115748 (Dr. W. Klohs, personal communication). The molecular basis for the differing activities of these four antifolates in the different MDR model systems is unclear at the present time.

Cell lines that possess the MDR phenotype rarely display cross-resistance to antimetabolites and alkylating agents [12, 17, 37, 42]. It is therefore of interest that H69AR cells are cross-resistant to Ara-C and melphalan (Fig. 4). Resistance to the latter drug has been associated with an increase in intracellular glutathione [15]. Since we have found that glutathione levels are diminished in H69AR cells [7], the modest (2-fold) but significant cross-resistance to this agent was particularly surprising.

In summary, we show that many features of the cross-resistance patterns of the H69AR cell line are similar to those obtained in other studies that have used MDR cell lines that overexpress P-glycoprotein. On the other hand, there were a number of unanticipated observations, viz., the cross-resistance to MRA-CN, bisantrene, PD 114541, ara-C, and melphalan. In addition, the complete lack of cross-resistance to the four lipophilic antifolates was unexpected. Taken together with results from other studies, our findings clearly illustrate the difficulty of drawing general conclusions about the efficacy of anticancer drugs, even within a given chemical class, against MDR cell lines. The basis of the variable cross-resistance patterns of different MDR cell lines is unknown and is probably complex. It is possible that the patterns of cross-resistance are related to the mechanism(s) underlying the resistance phenotype and, therefore, H69AR cells should not be expected to behave like cell lines that overexpress P-glycoprotein. Careful studies with cell lines in which it is known that only a *single* mechanism of resistance is functional are necessary to provide the data that would support or refute this idea. It is also possible that the intrinsic properties of tumours derived from a particular tissue might influence the cross-resistance profiles. In this regard, it would be of interest to determine cross-resistance patterns of a panel of MDR cell lines derived from the same tumour type.

Acknowledgements. The author wishes to thank Dr. Wayne Klohs for helpful discussions. The expert technical assistance of Debra Clements is gratefully acknowledged. Prof. R. Whitney is to be thanked for the preparation of some of the figures.

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