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Relationship between the Structure of Analogues of Amsacrine and their Degree of Cross-resistance to Adriamycin-resistant P388 Leukaemia Cells*

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Abstract—*A series of derivatives of* 9-*anilinoacridine related to the anti-leukaemia agent amsacrine have been tested in continuous exposure growth inhibition assays to determine the degree of cross-resistance in the Adriamycin-resistant P/ADR murine leukaemia line. Measured* IC_{50} *values for the two cell lines were only poorly correlated* (r = 0.51), *and cross-resistance as measured by the ratio of* IC_{50} *values varied from* 2-*fold and* 272-*fold. A high degree of resistance was found to be associated with the presence of amino or substituted amino groups on the acridine ring system. Logarithmic* IC_{50} *values were determined for other cell lines (*L1210 *leukaemia, Lewis lung carcinoma and HCT-*8 *human colon carcinoma) and were compared with those for the P*388 *lines to determine the degree of linear correlation. HCT-*8 *values were strongly correlated with P/ADR values* ($r = 0.84$) *while L*1210 *values correlated strongly with those of the sensitive P*388 *line* (r = 0.98). *Values for Lewis lung cells showed an intermediate pattern and correlated with a linear combination of values for both P*388 *lines* (r = 0.88). *Examination of available* IC_{50} *values for a number of rodent and human cell lines indicates that their sensitivity patterns are either P*388*-like or else intermediate between P*388 *and P/ADR. The series of amsacrine derivatives may be useful in characterizing the nature and degree of multidrug-resistance in cultured cell lines.*

INTRODUCTION

MULTIDRUG-resistance, a phenomenon where tumour cells, in acquiring resistance to an antibiotic or DNA-binding agent, concomitantly acquire resistance to a wide variety of other agents, has been intensively investigated in recent years [1, 2]. Resistance appears in many cases to be associated with an enhanced energy-dependent mechanism of drug efflux [3]. The acquisition of multidrug-resistance may result from increased gene expression or from gene amplification [4] and is usually associated with the appearance of one or more surface glycoproteins (P-glycloproteins) with a molecular weight of approx. 180 kD [5]. This type of resistance is at least partially overcome by the presence of verapamil and certain other compounds which are thought to interfere with the drug efflux mechanism [6]. A second type of resistance associated with a reduction in the activity of the enzyme DNA topoisomerase II has also been characterized [7].

Amsacrine, a derivative of acridine first synthesized by Cain and Atwell [8] and found to have clinically useful activity against some forms of acute leukaemia [9], is inactive against the Adriamycin-resistant P/ADR leukaemia line originally isolated by Johnson *et al.* [10] and shown to exhibit multidrug-resistance. Amsacrine has served as a basis in our laboratory for the synthesis of analogues with increased activity against solid tumours [11]. In addition to using *in vivo* solid tumour models for drug development [11, 12] we have developed assay methods based on microcultures in multi-well plates [13, 14] in order to determine whether culture data alone will identify analogues with superior antitumour properties. Extensive cell culture data have been presented previously for V79 Chinese hamster fibroblasts [15], L1210 leukaemia cells [13], P388 leukaemia cells [16], Lewis lung carcinoma cells [16], and a range of human tumour cell lines including HCT-8 carcinoma [14].

In the present study we have extended these

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studies to P/ADR cells in order to answer three questions. We wished to determine firstly whether the substitution pattern of amsacrine derivatives affected the degree of cross-resistance found in the multidrug-resistant line, secondly whether the pattern of activity found in the multidrug-resistant P/ADR line resembled any patterns of activity previously found in other cell lines which have not been selected for resistance, and thirdly whether amsacrine analogues could be designed which overcome multidrug-resistance. We have compared the activity of a range of amsacrine analogues, substituted in either the anilino or acridine parts of the molecule, against the P388 and P/ADR cell lines. The results support the hypothesis that some carcinoma cell lines have resistance mechanisms similar to those of the P/ADR line.

MATERIALS AND METHODS

Materials

All amsacrine analogues were synthesized in this laboratory [8] and were pure at the time of study as judged by thin layer chromatography.

Cell lines

The P388 leukaemia cell line was obtained in 1977 as a frozen stock from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, U.S.A. The P/ADR line was obtained as a frozen stock in 1980 from Mason Research Institute, Worcester, Massachussetts, U.S.A. Human cell lines were obtained as described previously [14]. The Colon 38 *in vivo* line was obtained from Mason Research Institute and passaged subcutaneously by standard methods. The tumour was disaggregated mechanically and cells adapted to tissue culture by Dr. W.R. Wilson, who kindly provided them.

Cell culture

The culture conditions for P388 (in ascorbate-containing medium) and for human cell lines has been described [16]. The procedure for P/ADR cells was similar to that already described for P388 cells. A sample of the frozen stock was thawed and 10^6 were inoculated intraperitoneally into DBA/2J mice. After 7 days, the peritoneal cells were removed in RPMI1640 growth medium containing heat inactivated foetal calf serum (10% v/v), sodium ascorbate (50 μM), mercaptoethanol (50 μM), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were diluted to 2×10^5 per ml and grown in T-flasks (10 ml volume) with frequent subculture for 2 weeks. Thereafter, the cells were used for growth inhibition studies, and were maintained for up to 12 weeks by subculture to 3×10^4 cells per ml in T-flasks (doubling time 12 h).

Growth inhibition assays were set up in 24-well trays (3×10^4 cells in 1 ml). Trays were incubated for 2 h before addition of drugs in 2 μl of 50% aqueous ethanol and further incubation at 37°C and 5% CO_2 in air for 70 h. The number of cells per well was determined using an electronic counter (Coulter Electronics). The IC_{50} value was defined as the nanomolar drug concentration required to reduce the cell density to 50% of that of the control cultures.

Growth of tumours in mice

P/ADR cells were passaged weekly in DBA/2J female mice by intraperitoneal injection of 10^6 cells. Cells were passaged by peritoneal washing with phosphate buffered saline [11]. For antitumour testing, B6D2F1 hybrid mice (DBA/2J male × C57BL/6J female) were inoculated i.p. with 10^6 P/ADR cells on day 0. Drugs were administered intraperitoneally to groups of 6 mice as solutions in 0.1 ml of 30% (v/v) aqueous ethanol on days 1, 5 and 9. Clinical drugs were dissolved in 5% dextrose–water. In each test a dose response profile was determined using dose increments of 1.5-fold for each drug. The optimal dose was defined as that giving the greatest life extension without showing toxicity. Deaths were recorded daily and the mean percentage of increase in lifespan was calculated with respect to that of 20 control mice [11].

RESULTS

Cross-resistance of P/ADR cells to amsacrine analogues

In vitro growth inhibition results for a series of anilino- and acridine-substituted analogues of amsacrine are listed in Table 1. It has been found that the presence of ascorbate in the growth medium affects the lifetime of amsacrine and its derivatives [14], although other derivatives not containing the anilino substitution pattern of amsacrine are less susceptible (unpublished data). In order to compare

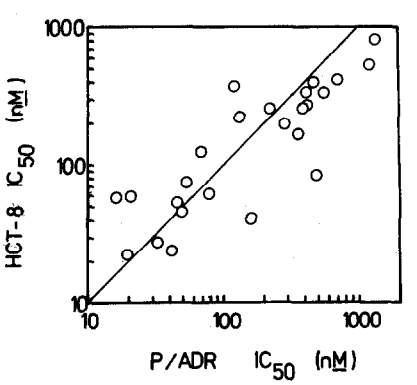
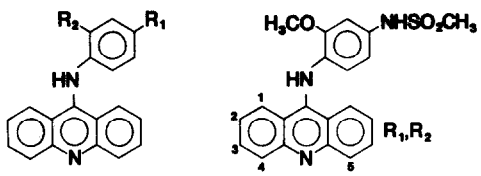


Fig. 1. Comparison of IC_{50} values for the multidrug-resistant line P/ADR with that of the human colon line HCT-8. Line represents equivalence points of IC_{50} values.

Table 1. In vitro *data (nanomolar* IC_{50} *values) for* 9-*anilinoacridine derivatives*



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No.	Formula	R_1	R_2	P/ADR	P388	Ratio	L1210	V-79	LLTC	HCT-8	Colon 38
1	I	$NHSO_2CH_3$	H	460 ± 100*	49 ± 5.7	9.4	38 ± 6	83	56	405 ± 34	—
2	I	$NHSO_2CH_3$	OCH_3	68 ± 4	12.5 ± 0.7	5.4	6.8 ± 1.2	16	26.5	127 ± 10	9.9 ± 1.2
3	I	$NHSO_2CH_3$	$NHCH_3$	350 ± 60	58 ± 6	6.0	71 ± 10	—	53	170 ± 17	43 ± 10
4	I	$NHSO_2CH_3$	$N(CH_3)_2$	390 ± 180	193 ± 38	2.0	520 ± 10	—	77	263 ± 38	—
5	I	$NHPO(OCH_3)_2$	H	420 ± 50	13 ± 2.2	32	16 ± 1.4	72	85	280 ± 40	—
6	I	$NHPO(OCH_3)_2$	OCH_3	1430 ± 70	23 ± 3.2	62	53 ± 17	60	73	830 ± 200	—
7	I	$NHPO(OCH_3)_2$	$NHCH_3$	404 ± 100	15 ± 3.5	27	21 ± 0.5	—	39	340 ± 110	—
8	I	$NHCOOCH_3$	H	130 ± 11	42 ± 7.7	3.1	51 ± 10	122	100	232 ± 24	—
9	I	$NHCOOCH_3$	OCH_3	220 ± 49	21 ± 2.1	10	40 ± 10	118	91	260 ± 40	—
10	I	$NHCOOCH_3$	$NHCH_3$	690 ± 170	176 ± 72	3.9	290 ± 26	—	121	600 ± 250	—
11	I	$NHSO_2C_6H_4NH_2$	H	41 ± 9	1.1 ± 0.5	37	0.8 ± 0.3	4.2	5.7	24 ± 2.2	—
12	I	$NHSO_2C_6H_4NH_2$	OCH_3	77 ± 33	2.6 ± 0.05	30	0.8 ± 0.6	—	7.6	62 ± 7	—
13	I	$NHSO_2C_6H_4NH_2$	$NHCH_3$	160 ± 40	5.7 ± 0.05	28	6.4 ± 1.4	—	10.3	41 ± 8	—
14	II	3-CH_3		19 ± 5	1.7 ± 0.4	11.2	1.6 ± 0.2	5.0	7.5	23 ± 1	—
15	II	3-OCH_3		48 ± 22	2.2 ± 0.2	22	2.1 ± 1.4	6.8	15.5	46 ± 11	—
16	II	3-Cl		20 ± 4	6.7 ± 1.0	3.0	3.9 ± 0.3	9.4	16.5	60 ± 10	6.5 ± 0.3
17	II	3-Br		33 ± 8	4.2 ± 0.2	7.9	3.3 ± 1.5	6.3	10.5	28 ± 5	2.5 ± 0.6
18	II	3-NH_2		290 ± 100	2.1 ± 0.4	138	1.6 ± 0.3	26	54	200 ± 70	—
19	II	3-$NHCH_3$		490 ± 200	1.8 ± 0.1	272	0.7 ± 0.06	18	23	82 ± 2	2.0 ± 0.6
20	II	3-$NHCOCH_3$		1220 ± 190	5.0 ± 0.7	244	4.3 ± 0.4	50	100	540 ± 40	—
21	II	3-$NHCOOCH_3$		560 ± 250	7.2 ± 1.5	78	7.2 ± 0.5	41	53	340 ± 120	8.6 ± 3.4
22	II	4-CH_3		52 ± 2	11.1 ± 0.2	4.7	6.4 ± 1.2	10.2	14.8	76 ± 7	—
23	II	4-OCH_3		16 ± 3	3.1 ± 0.1	5.2	2.5 ± 0.1	8.4	9.2	59 ± 9	—
24	II	4-$CONHCH_3$		120 ± 12	20 ± 0.3	6.2	16 ± 2.0	39	46	380 ± 40	—
25	II	4-CH_3	5-$CONHCH_3$	45 ± 13	4.9 ± 0.8	9.2	4.3 ± 1.6	9.5	12.6	54 ± 4	3.3 ± 0.9

*Mean ± standard error.

IC_{50} values of P388 and P/ADR cells (which grew poorly in alpha-modified minimal essential medium) with those of other cell lines, ascorbate was added to the medium.

The degree of cross-resistance shown by the P/ADR line was found to vary according to the substitution pattern of the anilinoacridine molecule. The highest degrees of cross-resistance were observed where the 1′-substituent was *p*-aminobenzenesulphonamide, or in the amsacrine series where the 3-substituent was amino or substituted amino.

Comparison of P/ADR and human colon HCT-8 carcinoma line

Further biological data were obtained for the HCT-8 human colon adenocarcinoma line so that it could be compared to P/ADR, and are presented in Table 1. Inspection of the data shows that the IC_{50} values for these lines are very similar (Fig. 1). A regression equation for the logarithmic IC_{50} values showed a higher degree of linear regression ($r = 0.84$) than that for P388 ($r = 0.69$).

Expression of IC_{50} *values as a function of* IC_{50} *values for P388 and P/ADR lines*

IC_{50} values for amsacrine derivatives using L1210 cells in ascorbate-containing media are presented in Table 1. These values are lower than those previously published for medium without ascorbate [13] but are very similar to those obtained for P388 cells. A small set of values for the Colon 38 line

Table 2. Regression equations relating logarithmic IC_{50} values of tumour cell lines to those of P388 and P/ADR leukaemia lines
$\text{Log}_{10} IC_{50} = a(\log_{10}IC_{50}\text{P388}) + b(\log_{10}IC_{50}\text{P/ADR}) + c$

Cell line	*a*	*b*	*c*	*r*
L1210	1.23 ± 0.06	—	−0.25	0.98
Jurkat	0.90 ± 0.11	—	0.05	0.78
V-79	0.56 ± 0.09	0.41 ± 0.09	0.00	0.91
LTCC	0.30 ± 0.08	0.43 ± 0.09	0.24	0.88
Co-38	0.95 ± 0.21	—	−0.03	0.91
HCT-8	0.27 ± 0.08	0.54 ± 0.09	0.68	0.90
HT-29	0.80 ± 0.12	0.51 ± 0.11	0.44	0.95
LoVo	1.11 ± 0.15	—	0.44	0.95
MCF-7	1.20 ± 0.15	—	0.52	0.96
MDA-231	0.95 ± 0.09	—	0.82	0.98
T-47D	1.23 ± 0.11	—	−0.02	0.98
MM-96	1.15 ± 0.07	—	0.44	0.99

(derived by growth of cells derived from Colon 38 solid tumours in mice) is also presented.

Previously published data for V79 Chinese hamster fibroblasts [15], Lewis lung carcinoma cells [16] and a number of human cancer cell lines [14] were analysed by regression analysis to determine the degree of similarity to the patterns of sensitivity shown by the P388 and P/ADR lines, and the results are shown in Table 2. As expected, IC_{50} values for the HCT-8 colon line show greater correspondence to those of the P/ADR line than that of the P388 line. The HT-29 human colon line shows somewhat less dependence, while the LoVo human colon line and the mouse Colon 38 line are adequately modelled by the P388 values alone. However, the constant term in the equation, which provides an indication of comparative sensitivities of the cell lines, indicates that the human colon lines are more resistant than the P388 line, whereas the Colon 38 line is of similar sensitivity.

Comparison of values for the Lewis lung carcinoma line show a sensitivity pattern intermediate between that of the P388 and P/ADR lines. The human melanoma, breast carcinoma and leukaemia lines all show the P388 type pattern of sensitivity. It is notable that Jurkat, L1210 and P388 cells have almost identical sensitivity. Other human T cell leukaemia lines also show similar sensitivity (unpublished results).

In vivo *activity of amsacrine analogues against P388-ADR leukaemia*

The series of amsacrine analogues chosen included compounds showing a high degree of *in vivo* antitumour activity. The results of *in vivo* testing are shown in Table 3. Many of the compounds for which a low degree of cross-resistance was found with the P/ADR line *in vitro* show significant activity against this line *in vivo*.

Table 3. In vivo *activity of amsacrine analogues against P/ADR leukaemia*

No.	Formula*	R_1	R_2	OD† (mg/kg)	ILS‡ (%)
1	I	$NHSO_2CH_3$		220	37
2	I	$NHSO_2CH_3$	OCH_3	13.3	0
3	I	$NHSO_2CH_3$	$NHCH_3$	13.3	0
4	I	$NHSO_2CH_3$	$N(CH_3)_2$	100	41
5	I	$NHPO(OCH_3)_2$		45	24
8	I	$NHCOOCH_3$		45	27
9	I	$NHCOOCH_3$	OCH_3	45	33
10	I	$NHCOOCH_3$	$NHCH_3$	45	50
11	I	$NHSO_2C_6H_4NH_2$		5.9	7
19	II	3-$NHCH_3$		3.9	21
21	II	3-$NHCOOCH_3$		3.9	3
25	II	4-CH_3	5-$CONHCH_3$	30	58

*Formula as in Table 1.
†Optimal dose/injection, administered days 1, 5, 9 after tumour inoculation.
‡Percentage increase in lifespan of treated animals.

DISCUSSION

The P/ADR cell line has been known for some time to exhibit the features of multidrug resistance [10]. For the compounds examined in Table 1, the degree of cross-resistance varies from 2-fold to 272-fold, indicating that small changes in drug structure can cause large changes in relative activity against the sensitive and resistant lines. For closely related derivatives of the 3′-demethoxy amsacrine derivative **1**, it has been found that amino substitution of the acridine ring increases the degree of resistance shown by the P/ADR line, and that this resistance is partially overcome by the addition of the calcium channel blocker verapamil [17]. Since verapamil had only a very small effect on the cross-resistance of compound **1** itself, and since verapamil is thought to reverse transport-mediated multidrug resistance, it is likely that the variations in cross-resistance observed in Table 1 result either from changes in the rate of uptake of compounds by cells, or by changes in the efficiency of outward drug transport of the P/ADR cell line.

The intracellular free drug concentration is maintained as a balance between uptake and efflux of drug. Both uptake and efflux of these compounds may depend on drug structure, accounting for the varying degrees of cross-resistance. An efficient outward-transport system, perhaps based on exocytosis [18], would be expected to act on free cytoplasmic drug. Compounds such as the carbamate **10**, which by binding strongly to DNA have a low cytoplasmic free drug concentration, may be transported less efficiently.

In a previous study utilizing cultures of LLTC mouse lung cells, it was concluded that amsacrine analogues showing high IC_{50} values for LLTC rela-

tive to P388 cells distributed poorly in mice [16]. Since these same compounds show high degrees of cross-resistance with the P/ADR cell line (Table 1), it is possible that there is a relationship between *in vivo* distribution and cellular uptake. One possible mechanism is that compounds such as the 3-amino and 3-substituted amino derivatives (compounds **18–21** in Table 1) traverse cellular membranes at a lower rate than do compounds such as amsacrine and CI-921 (compound **25**). It is known that amsacrine is taken up rapidly by mammalian cells [19] but uptake of amino derivatives has not been tested and may be slower.

Mechanisms of multidrug-resistance may pre-exist in cells unselected for multidrug-resistance [20]. In an attempt to answer the question of whether multidrug-resistance is evident in a range of rodent and human tumour lines, we have compared the sensitivity patterns of these cell lines with those of P388 and P/ADR cell lines. Using regression analysis, IC_{50} data sets can be expressed with a high degree of accuracy as a combination of data sets for the two P388 lines (Table 2). Data for the L1210 mouse leukaemia line and the Jurkat human leukaemia line are identical within the limits of experimental error to the P388 cell line. Data for the mouse Colon 38, human breast MCF-7, human breast T47-D, human melanoma MM-96 and human colon LoVo tumour lines are collinear with those for the P388 line. On the other hand, data for human colon HCT-8, human colon HT-29, mouse Lewis lung and Chinese hamster V79 fibroblast lines indicate a pattern intermediate between that of P388 and P/ADR, with the correspondence with the P/ADR line being greatest for HCT-8. This result suggests that these cell lines may have drug resistance mechanisms similar to those observed with the P/ADR cell line.

The IC_{50} values obtained here are related both to clonogenic data and to *in vivo* dose potency [16] suggesting physiological significance. Results of IC_{50} ratio determinations for P/ADR vs. P388 cells might identify specific compounds which could be screened for *in vivo* activity against the multidrug-resistant line. The carbamate derivative **10** shows significant *in vivo* activity (Table 3), and in view of the fact that further substitution of amsacrine (itself inactive in this tumour) resulted in CI-921 (compound **25**) which has significant activity, further compounds active against multidrug-resistant cells may be povided by the synthesis of derivatives of compound **10**. This is being investigated [21].

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