Eur J Cancer Clin Oncol, Vol. 20, No. 3, pp. 383–389, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

Differential Growth Inhibition of Cultured Mammalian Cells: Comparison of Clinical Antitumour Agents and Amsacrine Derivatives*

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Abstract—*The growth-inhibitory potencies in culture of a variety of clinical antitumour drugs have been compared using two widely studied cell lines: L1210 murine leukaemia and V79 Chinese hamster fibroblasts. Marked variations in the relative sensitivities of these two lines were observed with individual antimetabolites and DNA intercalating agents, while activities of alkylating agents and vinca alkaloids were similar for both cell lines. This experimental system was used to evaluate the possibility of designing intercalating drugs selective for a particular target cell. Results with derivatives of the antileukaemia agent amsacrine indicate that relative cytotoxicity can be modulated by simple monosubstitution within the 9-anilinoacridine ring system. The variation in ratios of inhibitory potencies within the latter series is similar to that observed for the structurally diverse group of clinically-utilized intercalators tested. These results suggest that amsacrine analogue development may provide agents having a different tumour spectrum and greater therapeutic utility than the parent drug.*

INTRODUCTION

ALTHOUGH selective toxicity is the basis of effective chemotherapy, the mechanisms responsible for the tumour selectivity of anticancer drugs are poorly understood. To some extent available agents may exploit differences in cytokinetic characteristics of tumour and normal tissue, or environmental features imposed by the microarchitecture of solid tumours such as low pH or hypoxia. However, in view of the marked differences in sensitivity of different cultured cell lines to certain drugs [e.g. 1–3], it is evident that important differences in intrinsic chemosensitivity between cell types do exist. These differences can be presumed to have an important influence on normal vs tumour cell discrimination *in vivo*, as well as on the spectrum of tumour types sensitive to any one cytotoxic drug.

This study was undertaken to evaluate the extent of cell line dependence in the cytotoxicity of 30 clinical agents representative of the major classes of antitumor drugs. The cell lines investigated, a murine leukaemia (L1210) and a spontaneously transformed Chinese hamster fibroblast line (V79–171b), have both been used extensively in studies of cytotoxic drug action in culture. Both lines are tumorigenic (see Materials and Methods). Our choice of V79 as a representative non-lymphoid cell line was influenced by the facility with which it can be maintained as a non-cycling plateau-phase population [4] or grown as multicellular spheroids [5, 6] to provide *in vitro* models of solid tumours. The method reported previously for assessing growth inhibition in drug-treated L1210 multi-well cultures [7] is here adapted for V79 cells to enable comparison of the two lines under similar culture conditions.

Previous studies in this laboratory [7, 8] have examined the sensitivity of cultured L1210 murine leukaemia cells to analogues of the intercalating agent amsacrine (*m*-AMSA, 4′-(9-acridinylamino)methanesulphon-*m*-anisidide). The latter compound has useful clinical activity

Accepted 30 August 1983.

*This work was supported by the Cancer Society of New Zealand, Inc., by its Auckland Division, and by the Medical Research Council of New Zealand.

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against human acute leukaemias [9], but little activity against the common solid tumours [10, 11]. We are seeking analogues of amsacrine with a broader spectrum of antitumour activity than is shown by the parent drug. We here compare the growth inhibitory potencies of a congeneric series of amsacrine analogues in L1210 and V79 cultures to learn whether cell type selectivity can be altered by structural modification within the constraints imposed by retention of the 9-anilinoacridine ring system.

MATERIALS AND METHODS

Drugs

Actinomycin D was purchased from the Sigma Chemical Company (St. Louis, MO, U.S.A.). Vincristine, vinblastine (Eli Lilly & Co., Indianapolis, IN, U.S.A.), etoposide (Bristol-Myers Co., New York, U.S.A.) and doxorubicin (Farmitalia, Milan, Italy) were purchased as the clinical formulations. All other clinical agents were generously supplied by the Research Laboratories of Warner-Lambert Company, Ann Arbor, MI, U.S.A. The latter drugs were analysed for purity before shipment under dry ice and desiccant, and stored subsequently under desiccant at -20°C. All congeners in the 9-anilinoacridine series have been synthesized previously in this laboratory [11, and references therein]. The purity of these agents was confirmed by TLC in at least two solvent systems before use. Stock solutions of drugs were prepared fresh for each experiment using the solvents shown in Table 1. Solutions prepared in PBS or H_2O were sterilized by filtration. All 9-

Table 1. ID_{50} *Values of agents in clinical use or in clinical trial*

Name	NSC No.	Solvent*	ID_{50} value (nM)† V79	ID_{50} value (nM)† L1210	Ratio‡	*P*§
Alkylating agents						
AZQ	182 986	A	309 ± 44	840 ± 175	0.37	<0.05
BCNU	409 962	A	5960 ± 580	10,300 ± 700	0.58	<0.05
Busulphan	750	B	>20,000	>16,000	—	—
CCNU	79 037	A	3340 ± 85	5700 ± 780	0.59	N.S.
Chlorambucil	3 088	A	13,150	9200 ± 1300	1.4	N.S.
Cisplatin	119 875	C	820 ± 17	460 ± 130	1.7	<0.05
Methyl CCNU	95 441	A	3150 ± 0	7100 ± 485	0.44	<0.05
Nitrogen mustard	762	A	635 ± 47	304 ± 3	2.1	<0.01
Thiotepa	6 348	A	2380 ± 370	2600 ± 530	0.92	N.S.
Antimetabolites						
Arabinosyl cystosine	63 878	A	240 ± 31	28 ± 6	8.6	<0.01
D-*O*-Norleucine	7 365	A	1000 ± 140	940 ± 280	1.1	N.S.
5-Fluorodeoxyuridine	27 640	A	4.6 ± 1.4	1.1 ± 0.5	4.2	<0.01
5-Fluorouracil	19 893	A	7900 ± 600	1300 ± 500	6.1	<0.01
Hydroxyurea	32 065	A	47,900 ± 11,600	47,000 ± 6500	1.0	N.S.
Methyl GAG	32 946	A	440 ± 13	260 ± 54	1.7	N.S.
Methotrexate	740	B	18.6 ± 0.8	18 ± 1.6	1.0	N.S.
6-Thioguanine	752	B	480 ± 0	27 ± 5	18	<0.01
Trimethoxyquin	22 745	A	6.0 ± 0	1.4 ± 0.05	4.3	<0.01
Antimitotics						
Etoposide (VP16)	141 540	D	274 ± 6	111 ± 49	2.5	N.S.
Vinblastine	49 842	D	2.2 ± 0.03	3.6 ± 0.7	0.61	N.S.
Vincristine	67 574	D	5.6 ± 1.0	7.6 ± 2.8	0.74	N.S.
DNA binding agents						
Actinomycin D	3 053	B	1.1 ± 0.18	0.33 ± 0.09	3.3	<0.05
Ametantrone	287 513	A	360 ± 58	52 ± 15	6.9	<0.01
Amsacrine	249 992	A	15.8 ± 0.5	33 ± 2.5	0.48	<0.01
Bisacridine	219 733	A	675 ± 170	110 ± 8	6.1	<0.05
Bisantrene	337 766	A	185 ± 5	15 ± 3	12.3	<0.01
Daunorubicin	82 151	A	18.3 ± 2.4	20 ± 2.6	0.88	N.S.
Doxorubicin	123 127	D	18.7 ± 2.5	30 ± 8	0.62	N.S.
Mitoxantrone	301 739	A	3.03 ± 0.31	0.75 ± 0.28	4.0	<0.01
Nitracrine	247 561	A	25 ± 4	22 ± 6	1.1	N.S.

*Solvents: A, EtOH: H_2O (1:1, v/v); B, DMSO: H_2O (1:1, v/v); C, PBS; D, H_2O.

†Mean ± standard error.

‡(ID_{50} for V79)/(ID_{50} for L1210).

§Level of significance of difference between ID_{50} values, as determined by Student's *t* test. N.S.: not significant at 5% level.

anilinoacridines (predominantly hydrochloride, methanesulphonate or isethionate salts) were dissolved in EtOH:H_2O (1:1, v/v) at 0.2–1 mM. Lactic acid (1.4 equivalents) was added to assist solubilization of free bases. Final concentrations of organic solvents in cultures did not exceed 0.5%, at which concentration no influence on cell growth was observed.

Cell lines

The establishment and maintenance of L1210 cell cultures in this laboratory has been described previously [7]. Briefly, these cells were grown in RPMI 1640 medium containing 10% v/v heat inactivated (56°C, 40 min) foetal calf serum (FCS), 2-mercaptoethanol (50 μM), penicillin (100 U/ml) and streptomycin (100 μg/ml) by subculturing three times per week (doubling time 12 hr). The L1210 cells were the same as used for *in vivo* testing in this laboratory [12].

V79-171b cells, obtained originally from Dr W. R. Inch, London, Ontario, Canada, were maintained in log-phase (doubling time 8.5 hr) in ribo- and deoxyribonucleoside-free ALPHA MEM containing 10% v/v heat-inactivated foetal calf serum without antibiotics by subculture to 10^4 cells/T-25 flask (Falcon) twice weekly. Single-cell suspensions were prepared by trypsinizing with 0.07% trypsin in citrate–saline (trisodium citrate, 4.4 g/l, KCl, 10 g/l; pH 7.3) for 10 min at room temperature and pipetting vigorously. Cultures were re-established from a frozen reference stock after not more than 30 passages. Cultures were free from mycoplasma as judged by cytochemical staining [12]. These cells were shown to be tumorigenic by subcutaneous inoculation of 8×10^6 cells into each of 6 BDF1 mice immunosuppressed by a modification of the method of Steel [13]. The tumour take rate was 100% with development of non-invasive, histologically anaplastic tumours at the site of injection. The mean latent period before tumours were palpable was 15 days, and the subsequent volume doubling time approximately 2–5 days.

Growth inhibition assays

Inhibition of L1210 cell growth was determined as described previously [7]. Briefly, 1-ml cultures were initiated in Nunclon 24-well culture dishes at 3×10^4 cells/ml in RMPI 1640 containing 10% heat-inactivated FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml), and drugs were added after 2 hr of incubation. Following growth for 70 hr in a CO_2 incubator at 37°C, corresponding to 5–6 doubling times for control cultures, cell density was determined using an electronic particle counter (Coulter Electronics). Each test included duplicate cultures at five two-fold dilutions of drug with two controls. The ID_{50} value was defined as that drug concentration which reduced the final cell density to 50% of the control value. L1210 ID_{50} values reported in this paper are averages, and include previously published data for some compounds [7, 8].

An analogous assay method was employed for V79 cells, with modifications to allow for differences in growth characteristics. Cultures (0.5 ml) containing 5×10^3 cells in ALPHA MEM with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml) were initiated in 24-well dishes. Addition of drugs, in 0.5 ml of the same medium, was delayed for 24 hr to allow attachment and resumption of exponential growth after trypsinization. At this time cultures contained $1.0–1.3 \times 10^4$ cells. Cell density was determined with a Coulter Counter 45 hr after drug addition (5–6 doubling times in the controls) by washing cultures once with PBS (NaCl, 8 g/l; KCl, 0.2 g/l; KH_2PO_4, 0.2 g/l; Na_2HPO_4, 1.15 g/l; $CaCl_2$, 0.1 g/l; $MgCl_2$, 0.1 g/l) and trypsinizing to prepare single-cell suspensions. In some experiments the clonogenicity after drug treatment was determined by plating cells in 60-mm diameter Petri dishes and incubating for 7 days before scoring colonies containing more than approximately 100 cells, as described in detail elsewhere [4].

RESULTS AND DISCUSSION

Characterization of V79 growth inhibition assay

Growth inhibition of V79-171b cells was evaluated by counting total substrate-attached cells after a drug exposure period corresponding to 5–6 doubling times in control cultures. The resulting dose–response curves were all similar in shape to the representative result for amsacrine illustrated in Fig. 1, and were similar to those for L1210 cells. Growth inhibition due to amsacrine was largely a result of irreversible cytotoxicity rather than reversible cytostatic effects, as shown by the marked loss of clonogenicity by 45 hr at concentrations in the vicinity of the ID_{50} (Fig. 1).

Repeat determinations of the ID_{50} of amsacrine in 47 separate experiments over a period of 28 months, using four different batches of FCS, provided a coefficient of variation (CV) of 20%, indicating the high reproducibility of the assay. The variations in ID_{50} were not significantly correlated with inter-experiment variations in the control growth rate. It should be noted that the measured cell densities in V79 cultures exclude cells which detach from the substrate as a result of drug treatment, whereas equivalent cells would be counted in the L1210 assays. However, for several compounds determination of the proportion of detached cells (which for amsacrine amounted to

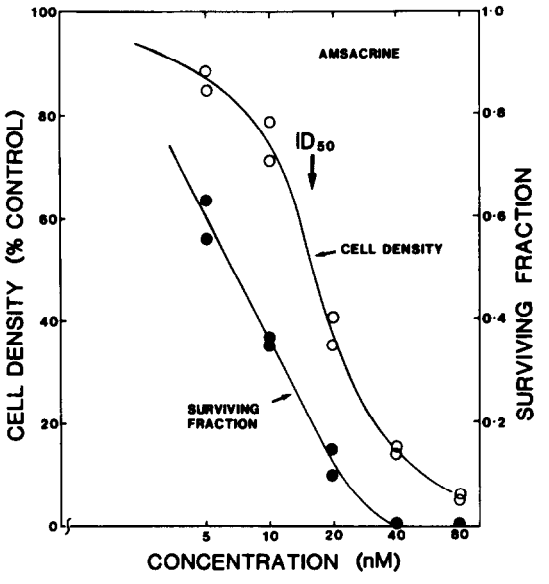


Fig. 1. Dose-response curves for inhibition of V79 cell growth after continuous exposure to amsacrine for 45 hr. Representative data for a single experiment are shown. Each point represents one culture. The total cell density (○) is normalized with respect to non-drug-treated controls (5.2×10^5 cells/ml in this experiment). The surviving fraction (○) is the plating efficiency of V79 cells after drug exposure relative to that for non-drug-treated controls (72% in this experiment).

approximately 1% of the total at the ID_{50} and 10% at a 4-fold higher concentration) indicated no significant change in ID_{50} if the latter was included. Cultures were always inspected by microscopy at the time of assay to ensure that the proportion of floating cells was low at concentrations in the vicinity of the ID_{50}.

Clinical antitumour agents

The growth inhibitory potencies of agents which are, or have been, in clinical use are shown in Table 1. For convenience, agents are listed under four categories (alkylating agents, antimetabolites, antimitotics and DNA binding agents), although the assignment of agents with poorly defined modes of action is somewhat arbitrary. Standard errors are shown in all cases where two or more determinations were made. The average number of determinations on each drug was 4.0 (excluding amsacrine, which was assayed many times). The average coefficient of variation for all drugs was 20% for V79 and 35% for L1210 assays. Therefore ID_{50} ratios (V79/L1210) of less than 0.6 or greater than 2 usually indicate a statistically significant difference in drug sensitivity.

All of the alkylating agents displayed similar cytotoxic potency against both V79 and L1210 cells (Table 1). Five of the 8 evaluable compounds provided ID_{50} ratios significantly different from unity, but these varied over only a 5-fold range (0.37–2.1). The three antimitotics again provided little indication of cell line selectivity, with ID_{50} ratios not significantly different from unity.

In contrast to the above, several antimetabolites provided clear indications of selective toxicity (Table 1), with an 18-fold range of ID_{50} ratios overall. Five of the 9 compounds evaluated showed ratios significantly different from unity, with values in the range 4.2–18, the highest selectivity being demonstrated by 6-thioguanine. It is noteworthy that all 5 agents showing significant selectivity were more active against L1210 than V79. That the antimetabolites show relatively pronounced selective toxicity is consistent with their expected sensitivity to differences in intermediary metabolism (metabolite pool sizes, quantitative and qualitative differences in target enzymes) in different cell types.

It is perhaps more remarkable that the DNA binding drugs in Table 1, all of which bind reversibly to DNA by intercalation, show marked cell type selectivity with a 25-fold range of ID_{50} ratios (0.48–12.3). Six of the 9 agents tested demonstrated significant selectivity. In fact, there is considerable evidence that one of the intercalators with a ratio close to unity, nitracrine, acts through covalent inter-strand cross-linking of DNA rather than via reversible binding to DNA [14, 15], and could thus be classed as an alkylating agent. The other two agents with ratios close to unity, the anthracycline antibiotics

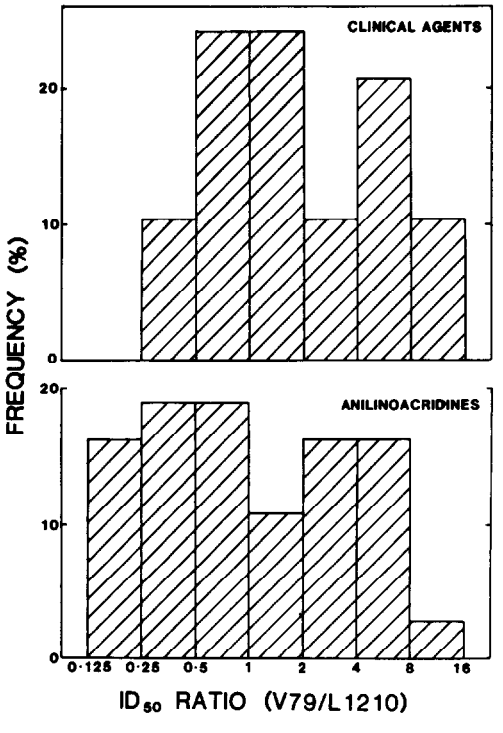


Fig. 2. Distribution of ID_{50} ratios for 29 clinical antitumour drugs (Table 1, excluding amsacrine; top panel) and 37 anilinoacridines (Tables 2 and 3; bottom panel).

doxorubicin and daunorubicin, may also act via DNA-reactive oxidative metabolites [16] rather than through intercalative binding *per se*. Since the mechanism of cytotoxicity of intercalators has not been clearly established, the cause of the marked cell type selectivity of these agents is uncertain. It is unlikely that intercalative binding will have sufficient selectivity to account for the differences in cytotoxicity. One view gaining increasing support is that intercalators induce DNA damage through interference with the action of DNA topoisomerases [17]. Although the relationship of intercalator-induced DNA lesions to cytotoxicity remains to be elucidated, it is possible that these drugs interact in a specific manner with DNA-associated enzymes, such as topoisomerases, and that variations in such targets in different cell types might account for a part of the observed selectivity. However, we have not excluded other explanations such as differential transport or metabolism of drugs.

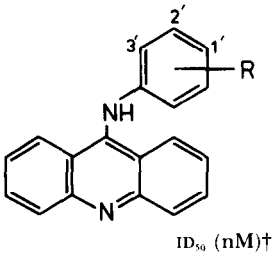
Amsacrine analogues

The above observations of pronounced cell type selectivity among DNA binding drugs prompted us to examine a congeneric series of analogues of amsacrine. The ID_{50} values in Tables 2 and 3 represent all previously described 9-anilinoacridine derivatives which have been tested against both V79 and L1210, without any selection of compounds after obtaining the data. The 1'-substituted derivatives of 9-anilinoacridine (Table 2) provide ID_{50} ratios consistently above unity, indicating selectivity for L1210 cells. For most agents this difference is statistically significant. For the 3, 6-diamino-1'methanesulphonamide derivative (compound 13) the ratio is high as 12. Introduction of a 3'-OCH_3 group into the 1'methanesulphonamide derivative to give amsacrine (Table 3, compound 17) lowered the ratio to 0.49.

The majority of acridine-substituted amsacrine analogues retain this low ratio (Table 3), but again significant variations in selectivity are seen within this group of compounds, with ratios ranging from 0.13 (compounds 28 and 29) to 4.7 (compounds 23). Of all the 9-anilinoacridines tested, 59% have ratios significantly different from unity, with an almost 100-fold range of 0.13–12. Thus the cell line selectivity achievable within this congeneric series is at least as great as that observed for the structurally diverse group of intercalators in clinical use.

Beyond the above generalizations we cannot specify the drug structural features of amsacrine analogues which influence relative activity towards V79 and L1210 cells. Linear regressions between the logarithms of the ID_{50} ratios for

Table 2. ID_{50} *Values of substituted 9-anilinoacridines*



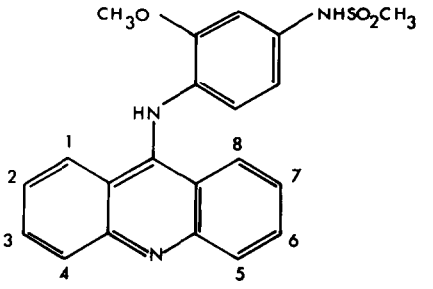
No.	Substitutents	ID_{50} (nM)† V79	L1210	Ratio‡	*P*§
1	Parent	4250 ± 650	3400 ± 830	1.25	N.S.
2	1'NH_2	705 ± 150	470 ± 120	1.5	N.S.
3	1'$NHCH_3$	400	243 ± 32	1.65	N.S.
4	1'$NHCOCH_3$	410	210 ± 66	1.95	N.S.
5	1'$NHCOOCH_3$	122 ± 12	51 ± 10	2.4	<0.01
6	1'$NHCOOCH_3$, 3'OCH_3	118 ± 12	40 ± 10	2.95	<0.01
7	1'$NHCONHCH_3$	640	110 ± 5	5.82	<0.01
8	1'$NHCONHC_6H_5$	18 ± 4	3.3 ± 1.2	5.45	<0.01
9	1'$NHSO_2C_6H_5$	10.8 ± 2.2	4.4 ± 0.8	2.5	<0.05
10	1'$NHSO_2C_6H_4$ *p*NH_2	4.2 ± 0.2	0.8 ± 0.3	5.3	<0.01
11	1'$NHSO_2CH_3$	83 ± 5	38 ± 6	2.2	<0.01
12	1'$NHSO_2CH_3$, 2'OCH_3	1800 ± 60	660 ± 120	2.7	<0.01
13	1'$NHSO_2CH_3$ 3,6 diNH_2	82 ± 6	6.7 ± 3	12	<0.01
14	2'$NHSO_2CH_3$	465 ± 15	94 ± 16	4.95	<0.01
15	1'$NHPO(OCH_3)_2$	72 ± 8	16 ± 1.4	4.5	<0.01
16	1'$NHPO(OCH_3)_2$, 3'OCH_3	60 ± 6	70	0.86	N.S.

†Mean ± standard error.

‡(ID_{50} for V79)/(ID_{50} for L1210).

§Level of significance of difference between ID_{50} values, as determined by Student's *t* test. N.S.: not significant at 5% level.

Table 3. ID_{50} *Values of substituted 9-anilinoacridines*



No.	Substituent	ID_{50} (nM)† V79	L1210	Ratio‡	P§
17	Parent	15.8 ± 0.5	32 ± 2.5	0.49	<0.01
18	2-CH_3	1300 ± 145	1400	0.93	N.S.
19	2-NH_2	21 ± 1	78 ± 3	0.28	<0.01
20	2-OCH_3	3600 ± 400	4120	0.87	N.S.
21	3-CH_3	5.0 ± 0.7	12 ± 1.7	0.42	N.S.
22	3-CH_2CH_3	37 ± 3	110 ± 40	0.34	N.S.
23	3-NH_2	26 ± 4	5.5 ± 0.5	4.7	<0.01
24	3-$NHCH_3$	18 ± 2.4	6.0 ± 0.6	3.0	<0.01
25	3-$NHCOCH_3$	50	67 ± 3.5	0.75	N.S.
26	3-$NHCOOCH_3$	41 ± 1.5	41 ± 2.5	1.0	N.S.
27	3-OCH_3	6.8 ± 1.6	22 ± 3.0	0.31	<0.05
28	3-Cl	9.4 ± 0.6	71 ± 2.5	0.13	<0.01
29	3-Br	6.3 ± 0.8	49 ± 8.7	0.13	<0.01
30	3-I	3.3 ± 0.3	15 ± 7.6	0.22	N.S.
31	3-NO_2	51 ± 3.4	108 ± 9	0.47	<0.01
32	3-CF_3	280 ± 56	480 ± 100	0.58	N.S.
33	3-$N_3(CH_3)_2$	7.7 ± 1.4	15 ± 3.4	0.51	N.S.
34	4-CH_3	10.2 ± 1.3	30 ± 5.3	0.34	<0.01
35	4-OCH_3	8.4 ± 0.5	40 ± 10	0.21	<0.05
36	4-$CONH_2$	34 ± 3	220 ± 66	0.15	<0.01
37	4-$CONHCH_3$	39 ± 3	200 ± 61	0.20	N.S.

†Mean ± standard error.

‡(ID_{50} for V79)/(ID_{50} for L1210).

§Level of significance of difference between ID_{50} values, as determined by Student's *t* test. N.S.: not significant at 5% level.

derivatives in Table 3 and published [11] physiocochemical data for lipophilicity, DNA association constant and acridine base strength show that the latter parameter (pK_a) is weakly but significantly correlated:

$$\log (ID_{50} \text{ ratio}) = 0.13\ pK_a - 1.29$$

($r = 0.60$, $P < 0.01$). This could indicate a role for the uncharged form of the drug in transport, with the most highly charged compounds being excluded from V79 cells to a greater extent than from L1210 cells. However, the present data base includes too few compounds with ratios distant from unity to warrant a full multivariate correlative study.

It is evident that simple monosubstitution in the 9-anilinoacridines can markedly alter selective toxicity. This observation underlines the critical importance of employing *in vitro* screening systems which reflect the required target population *in vivo*. Thus although L1210 growth inhibition assays have been used successfully to predict *in vivo* activity of amsacrine analogues against this leukaemia [7], the *in vitro* sensitivity of L1210 is unlikely to be a valid predictor of activity against other tumours.

In a series of amsacrine congeners, structural requirements for *in vivo* activity against P388 leukaemia and Lewis lung carcinoma have been found to differ [18]. It is notable that of the 16 compounds in Table 3 that have been tested *in vivo* against the Lewis lung carcinoma all with ratios of greater than 0.5 are inactive, and the most active compounds (28, 29, 35 and 37) have ratios of less than 0.25.

Recent studies in this laboratory [Finlay, personal communication] have indicated that amsacrine has a shorter half-life in RPMI 1640 (used for L1210 cell culture) than in ALPHA MEM (used for V79 cells) and that this differential stability may not be the same for all amsacrine analogues. Variations in drug half-life in culture medium would complicate interpretation of the differences in ID_{50} ratio observed in this study.

However, the apparent correlation between this ratio and relative activity against the Lewis lung tumor suggests that the ID_{50} ratio may be a useful indicator of drug selectivity.

In an extension of the approach described here, we are currently attempting to identify amsacrine analogues with altered tumour spectrum by testing agents against a panel of mouse and human carcinoma-derived cell lines.

Acknowledgements—The authors wish to thank Drs W. A. Denny, G. J. Atwell and G. W. Rewcastle for resynthesis of some of the amsacrine analogues, Ms Rosalee Nash for carrying out the L1210 culture work and Mrs Sally Hill for typing the manuscript.

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