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Comparison of *in Vitro* Activity of Cytotoxic Drugs Towards Human Carcinoma and Leukaemia Cell Lines*

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Abstract—*Eight human haematopoietic cell lines and four human carcinoma lines were used to compare the activity of a number of cytotoxic drugs including amsacrine, the amsacrine analogue CI-921, methotrexate, nitracrine, doxorubicin, daunorubicin and 5-fluorouracil. Activity was assessed by means of semiautomated microculture growth inhibition assays. Cell density of the non-adherent cell lines was measured using the technique of Mosmann* (J Immunol Methods 1983, **65**, 55–63), *in which the dye thiazolyl blue (MTT) is metabolised to a dark blue formazan product. This technique gives similar results to those obtained by direct cell counting in an electronic cell counter, and when applied to some adherent cell lines gives similar results to those obtained by the methylene blue staining technique previously developed* (Anal Biochem 1984, **139**, 272–277). *Both methylene blue and MTT methods were used to investigate cytotoxicity in conjunction with semi-automated 96-well microculture plate techniques. The results show that the three T-cell leukaemia lines (CCRF-CEM, Jurkat and MOLT-4) are more sensitive to DNA-binding drugs (excluding nitracrine) than are the colon carcinoma lines (HCT-8, HT-29, SW480 and SW620). The more resistant haematopoietic lines are intermediate in drug sensitivity between the T cell leukaemia and carcinoma lines. The DNA binding drugs show remarkably similar patterns of differential activity against the different cell lines.*

INTRODUCTION

DESPITE considerable advances in our understanding of cancer, the chemotherapy of solid tumours remains a major clinical problem. New methods need to be developed which allow the identification of cytotoxic agents with increased selectivity towards carcinomas. It can be argued that mouse leukaemias, which are commonly used in drug development and discovery programmes, will select those drugs which are active against haematopoietic elements. This may in part explain why the destruction of haematopoietic tissue is one of the most common types of dose-limiting toxicity in current chemotherapy.

In order to design agents with improved cytotoxicity towards solid tumours relative to haematopoietic cells, several areas of difficulty need to be addressed. These include cytokinetic barriers to drug action, the distribution of drugs within solid tumours, and the relative inherent drug sensitivities of solid tumour and haematopoietic cells.

Growth inhibition assays using cultured cell lines may be able to identify drugs which show selectivity for cells of different histological types. For a range of several DNA intercalating drugs including derivatives of the clinical antileukaemia agent amsacrine [1] it has been shown that a leukaemia cell line was generally more sensitive than carcinoma lines [2]. Of six human carcinoma lines, those derived from colon tumours were the most resistant. In a study using murine cell lines, those derivatives of amsacrine with the highest activity in culture towards carcinoma cells relative to the leukaemia cell line showed the greatest activity towards the Lewis lung tumour, an experimental carcinoma in mice (Baguley and Wilson, in preparation). The derivative with a particularly high relative activity (CI-921 see Fig. 2 for structure) is a candidate drug for clinical trial [3].

The use of both leukaemia and carcinoma cell lines *in vitro* may thus be of use in identifying compounds which are intrinsically more toxic for carcinoma cells than leukaemia cells or the normal blood cells from which they are derived. This

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report describes the use of a panel of human leukaemia and carcinoma cell lines to define the spectrum of cytotoxicity of several anticancer agents. To facilitate this, a semiautomated assay was developed to quantitate growth inhibition of non-adherent cell lines. The use of microtitre plate technology has been shown to be an efficient and precise method of quantitating cell protein [4]. We have exploited this approach to develop a semi-automated microculture growth inhibition assay utilizing adherent cell lines [5]. The validity of this method has been established recently by the demonstration that growth inhibition assays using microculture plates correlate well with clonogenicity assays [6]. The recent description of a quantitative staining method for living cells [7] has now permitted the adaptation of this method to non-adherent cell lines. The results indicate that several intercalating agents show the same spectrum of activity against a panel of haematopoietic and epithelial neoplastic cell lines. This stresses the necessity for searching for new intercalating compounds which are selectively cytotoxic for carcinoma cells.

MATERIALS AND METHODS

All cells are of human origin. Leukaemia cell lines used in this study are listed in Table 1. They were passaged twice weekly in 100 ml bottles using 25 ml of growth medium. The colon carcinoma cell lines used were HCT-8 (supplied by the Warner-Lambert Co., Ann Arbor, Michigan), HT-29 (provided by Dr. J. Fogh, Sloan–Kettering Institute, N.Y.) and SW480 and SW620 (both from the American Type Culture Collection). The carcinoma cells were cultured in 25 cm^2 flasks and passaged weekly using trypsin (0.1%, Difco) in citrate saline (trisodium citrate dihydrate 4.4 g/l, KCl 10 g/l, pH 7.3). All cells were cultured in Alpha MEM (Gibco, Grand Island, N.Y.) supplemented with foetal calf serum (Smith Biolab Ltd., New Zealand) (10% v/v except for HL-60 which required 20%) and glutamine (2mM). For cytotoxicity assays, penicillin (100 units/ml) and streptomycin (100 μg/ml) were also added.

Drug sensitivity assays

Assays defined the IC_{50} value as the concentration of drug which inhibits cell growth to 50% of that in control (drug-free) cultures. Growth was determined by counting cells, by colorimetric analysis using methylene blue (Ajax Chemical Co., Sydney, Australia), or by analysis using (4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT thiazolyl blue; Sigma). Assay procedures are outlined in Fig. 1.

For non-adherent leukaemia lines, cell growth was quantitated by the method of Mosmann [7]. Cultures in microculture plates (Linbro) were established in 135 μl volumes (at the cell concentrations shown in Table 1) and incubated for 4 hr prior to adding cytotoxic compounds diluted in growth medium. Dilution series of cytotoxic compounds were prepared in separate microculture plates (five concentrations encompassing a 16-fold range). Solutions of drugs, at 10 × the required concentration were added to the cultures in 15 μl volumes. Control cultures received medium alone. Cultures were incubated for 4 or 5 days. Growth of some cell lines (Table 1) was improved if the culture plates were tilted (2°) so that the cells collected on one side of the well. To determine cell

Table 1. Leukaemia/lymphoma lines used in this study

Cell line	Tumour type†	Obtained from	IC_{50} assay			
			Cells/well	Plate tilted	Duration of drug exposure	Duration of MTT reaction*
CCRF-CEM	T-ALL	ATCC	3,750	Yes	4 days	4 hr
Jurkat	T-ALL	Prof. J. Watson Auckland	3,750	No	4 days	2 hr
MOLT-4	T-ALL	ATCC	3,750	Yes	4 days	4 hr
Daudi	Burkitt's lymphoma	ATCC	7,500	Yes	5 days	4 hr
Raji	Burkitt's lymphoma	ATCC	3,750	No	5 days	2 hr
U937	Histiocytic lymphoma	ATCC	3,750	Yes	4 days	2 hr
HL-60	APL	ATCC	3,750	Yes	5 days	4 hr
K562	CML	ATCC	3,750	No	5 days	2 hr

* Unless stated otherwise

† T-ALL: acute lymphatic leukaemia, T cell type; APL: acute promyelocytic leukaemia; CML chronic myelogenous leukaemia.

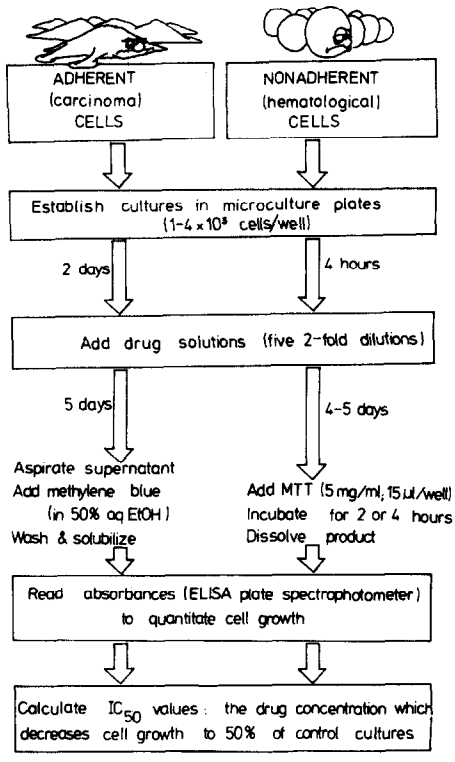

Fig. 1. Flow diagram for semiautomated microculture assays giving IC_{50} *values of cytotoxic compounds in cultures of adherent and non-adherent cell lines.*

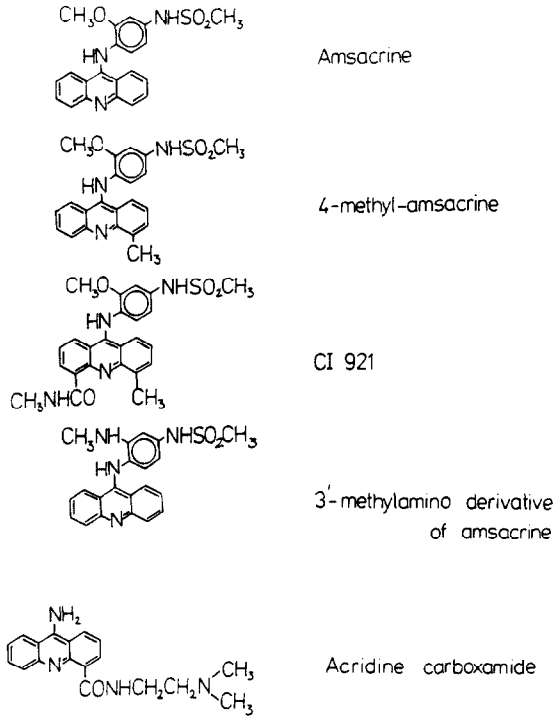

Fig. 2. Structures of amsacrine, CI-921 and experimental antineoplastic agents used in this report.

growth, MTT (5 mg/ml) in phosphate-buffered saline [2] was added to each culture (15 µl/well), and the cultures incubated at 37°C for another 2 or 4 hr (Table 1). The dark-coloured crystals which were produced by viable cells were then solubilized by vigorous pipetting after addition of 2-propanol containing 4×10^{-2}M HCl (165 µl/well). Absorbances were determined using a Titertek Multiskan ELISA plate spectrophotometer (Flow Laboratories) using a sample wavelength of 540 nm and a reference of 690 nm. The absorbance of wells containing cells was compared to those in which MTT was incubated with growth medium alone.

For the adherent colon carcinoma lines, cells were cultured in microculture plates and growth quantitated by the binding of methylene blue to cell monolayers. Methylene blue binding was determined using an ELISA plate reader, as described [5]. Cultures were initiated at 1×10^3 (HCT-8, HT-29) or 4×10^3 (SW480, SW620) cells per well (135 µl/well). After 2 days, drug at 10 × the required concentration in growth medium (15 µl) was added. Cell density was determined 5 days later.

Sources of the clinical and experimental cytotoxic agents used and preparation of stock solutions have been described [2]. Structures of amsacrine, CI-921 and experimental acridine compounds are given in Fig. 2.

RESULTS

Assay of leukaemia cell growth in microculture plates using MTT

Cells from four different human leukaemia cell lines were grown in 96-well microculture plates in the presence of three different concentrations of the DNA intercalating drug CI-921 (structure in Fig. 2). Cell growth was determined in representative cultures either by counting cells with a haemocytometer, or by means of the colorimetric method of Mosmann, in which the time of incubation with MTT was varied from 0.5 to 5 hr. The rate of colour development was dependent on the cell line, and in each case the kinetics were non-linear with time, both in control cultures and those where cell number has been greatly diminished by cytotoxic drug (Fig. 3). However, IC_{50} values calculated from these data were largely independent of the duration of the incubation with MTT (Fig. 4). IC_{50} values were slightly higher when determined by the colorimetric assay than by cell number but the differences were not greater than 2-fold (Fig. 4).

Determination of IC_{50} *values using different assays for cell density*

The relationship between cell number and intensity of staining was investigated further for several drugs by counting one half of the contents of each well (Coulter Counter) and performing the MTT assay on the cells remaining. As shown in Fig. 5, IC_{50} values determined by the colorimetric

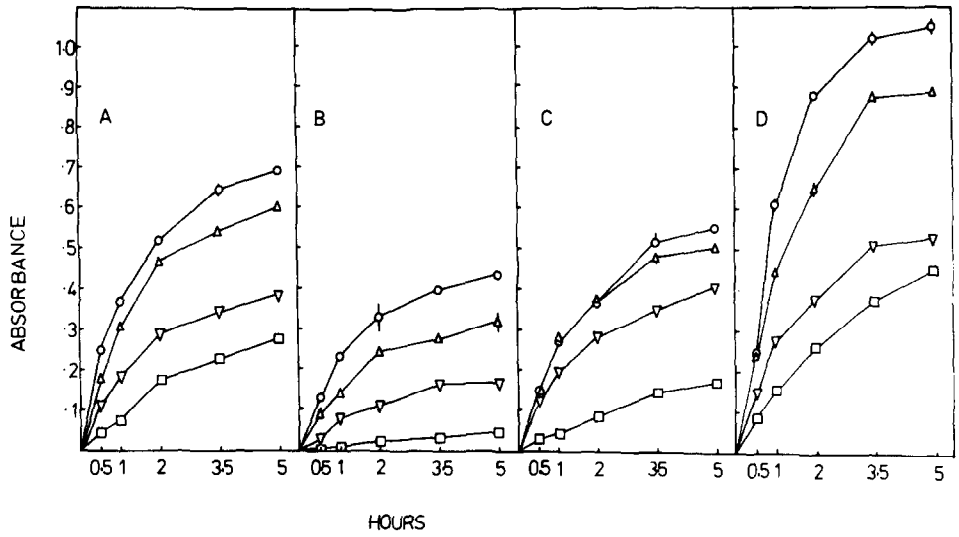

Fig. 3. Kinetics of colour development in cultures of human leukaemia cells. Cells were cultured in microculture plates in the absence of presence of CI-921 as described, except that eight cultures were used for each point. Cells were incubated with MTT for the time indicated on the abscissa. Cell lines, the drug concentrations used, and the number of cells recovered from representative cultures (means of three cultures ± S.E.) are:

A. *Jurkat cells*:	(○) *control*,	$9.4 \pm 0.1 \times 10^4$;	C. *U937 cells*:	(○) *control*,	$10.5 \pm 1.6 \times 10^4$;
	(△) 3.1 nM,	$5.8 \pm 0.5 \times 10^4$		(△) 6.25 nM,	$7.4 \pm 1.0 \times 10^4$
	(▽) 6.25 nM,	$2.0 \pm 0.3 \times 10^4$;		(▽) 12.5 nM,	$4.0 \pm 1.0 \times 10^4$
	(□) 12.5 nM,	$0.9 \pm 0.1 \times 10^4$;		(□) 25 nM,	$0.8 \pm 0.2 \times 10^4$;
B. *MOLT-4 cells*:	(○) *control*,	$10.4 \pm 0.3 \times 10^4$;	D. *K562 cells*:	(○) *control*,	$10.9 \pm 0.9 \times 10^4$;
	(△) 3.1nM,	$7.0 \pm 0.9 \times 10^4$;		(△) 12.5 nM,	$6.7 \pm 0.2 \times 10^4$;
	(▽) 6.25 nM,	$1.9 \pm 0.1 \times 10^4$;		(▽) 25 nM,	$3.0 \pm 0.4 \times 10^4$;
	(□) 12.5 nM,	$0.2 \pm 0.1 \times 10_4$;		(□) 50 nM,	$1.3 \pm 0.2 \times 10^4$.

S.E. bars are indicated where they exceed the size of the symbol.

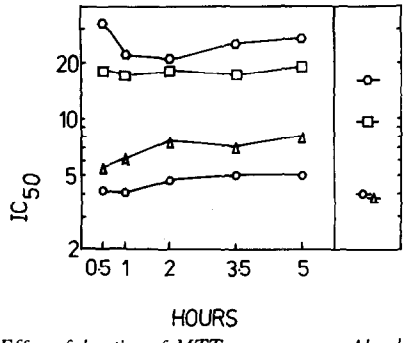

Fig. 4. Effect of duration of MTT assay on IC_{50}. Absorbance values and cell numbers presented in Fig. 3. were used to obtain IC_{50} values for Jurkat (△), MOLT-4 (○), U937 (□), and K562 (⬭) cells. The right hand panel represents the IC_{50} values derived from counting cells (haemocytometer) in representative wells.

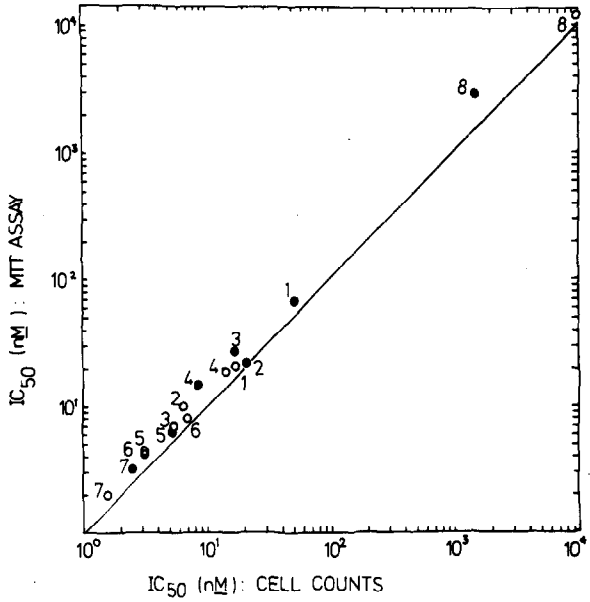

Fig. 5. Comparison of IC_{50} values obtained by the MTT assay and by cell counts. IC_{50} assays were performed on Jurkat (○) and U937 (●) cells. Seventy-five microlitres of each culture were removed for cell counting (Coulter electronic particle counter) and the remainder assayed by the MTT procedure for 2 (Jurkat) or 5 (U937) hr. Drugs are numbered as follows: 1. Amsacrine; 2. CI-921; 3. acridine carboxamide; 4. methotrexate; 5. nitracrine; 6. doxorubicin; 7. daunorubicin; 8. 5-fluorouracil.

assay are fractionally (1.5 times) higher than those determined by cell counting, and different agents show the same relative potency.

When the IC_{50} values of carcinoma cells were determined by the methylene blue staining method and the MTT colorimetric assay, an excellent correlation was obtained using SW480 and SW620 cells (Fig. 6). However, HCT-8 cells metabolized MTT in an anomalous fashion; control cells which had grown to confluence under the conditions of the assay metabolised MTT more slowly than did the cells present in cultures treated with CI-921 at the IC_{50} (data not shown). For the IC_{50} assays

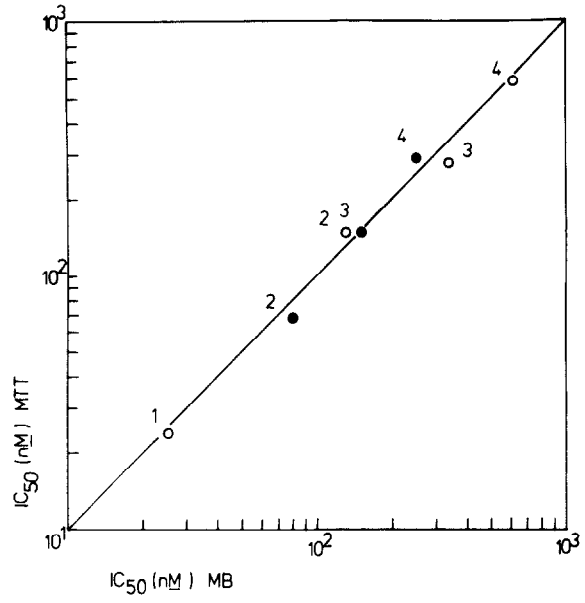

Fig. 6. Comparison of IC_{50} *values obtained by the MTT assay and by methylene blue staining of cultures.* IC_{50} *assays were performed with SW* 480 (○) *and SW* 620 (●) *colon adenocarcinoma cells using the MTT and methylene blue colorimetric assays in parallel cultures. Drugs used were, daunorubicin* (1), *acridine carboxamide* (2), *amsacrine* (3), *and the* 3′*-methylamino derivative of amsacrine* (4).

described in the next section, the MTT procedure was used for haematopoietic lines, and the methylene blue procedure for carcinoma lines.

Determination of IC_{50} *values for a range of cytotoxic agents*

IC_{50} values were determined for several clinical and experimental anticancer drugs using eight leukaemia and four colon carcinoma cell lines (Table 2 and Fig. 7). For the two anthracyclines tested (daunorubicin, doxorubicin) cell lines derived from lymphoid tumours (T-cell acute leukaemia and Burkitt's lymphoma) comprised the most sensitive group. The other three haemato-

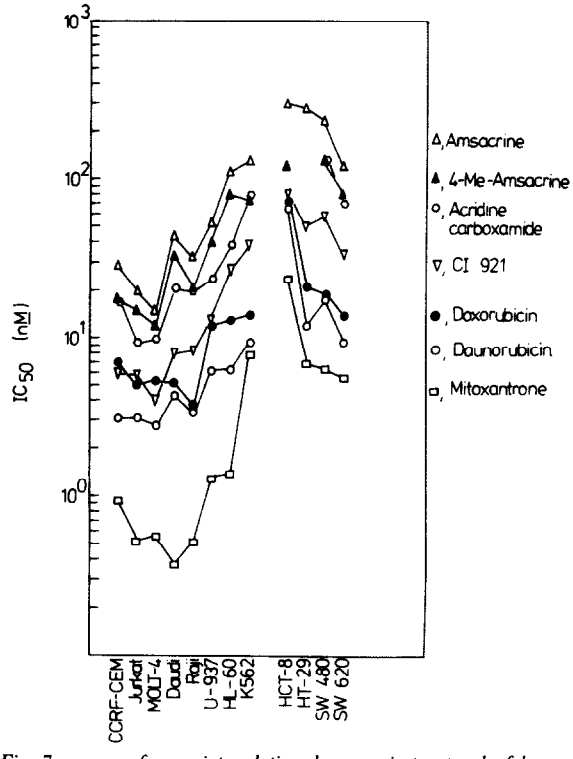

Fig. 7. IC_{50}*s of seven intercalating drugs against a panel of human tumour cell lines.*

Table 2. IC_{50} *values (nM) of* 10 *cytotoxic drugs*

	T-ALL			BL		HL	APL	CML	COLON CARCINOMA			
Agent	CCRF-CEM	Jurkat	MOLT-4	Daudi	Raji	U937	HL-60	K562	HCT-8	HT-29	SW480	SW620
Daunorubicin	3.1±0.2 (4)	3.1±0.4 (4)	2.8±0.1 (3)	4.3±1.2 (3)	3.4	6.3±0.1 (4)	6.3±0.1 (4)	9.5±0.3 (4)	66±7 (2)	12±2 (3)	18±2 (4)	9.4±0.3 (4)
Doxorubicin	7.2±1.3 (3)	5.0±0.7 (3)	5.4±0.3 (2)	5.3±2.2 (2)	3.8	12±1.2 (3)	13±1 (3)	14±1 (3)	72	21±1 (2)	19±2 (3)	14±0 (3)
Mitoxantrone	0.93	0.52	0.57	0.37	0.52	1.3	1.4	7.9	24	6.9	6.4	5.6
Amsacrine	29±4 (13)	20±1 (13)	15±1 (11)	44±4 (5)	32±1 (5)	53±3 (13)	110±10 (9)	130±10 (9)	300±60 (3)	280±70 (3)	230±30 (11)	120±10 (13)
4-Methyl amsacrine	18±6 (3)	15±2 (3)	12±2 (3)	33±8 (2)	21±2 (2)	40±9 (3)	80±0 (2)	74±8 (2)	120	–	130±50 (3)	81±4 (3)
CI-921	6.0±0.6 (6)	5.8±0.8 (6)	4.0±0.4 (4)	7.9	8.3±0.3 (3)	13±1 (6)	27±4 (5)	38±3 (5)	81±9 (3)	50±12 (3)	58±10 (4)	33±5 (4)
Acridine carboxamide	17±1 (2)	9.5±0.6 (2)	9.9±0.2 (2)	21±1 (2)	20	24±3 (3)	39±2 (2)	80±4 (2)	–	–	130±30 (3)	69±7 (4)
Methotrexate	26±5 (2)	24±4 (2)	40	–	22±2 (2)	17±5 (2)	26	16±4 (2)	45	37	20	37±8 (2)
5-Fluourouracil	3200±200 (2)	4500±800 (2)	2200	6900	4200±700 (2)	2400±600 (2)	2900±100 (2)	7700±100 (2)	3700	2000	3600	6600±300 (2)
Nitracrine	13±2 (2)	6.7±0.3 (2)	2.4±0.2 (2)	9.9±0.2 (2)	12±1 (2)	10±2 (2)	23±1 (2)	10±3 (2)	7.6	8.8	18±4 (2)	9.7±1.4 (2)

Values represent arithmetic means ± S.E.
Numbers in parentheses indicate the numbers of independent determinations (if greater than 1).

poietic cell lines were of intermediate sensitivity. The colon carcinoma lines were the most resistant group. The activity spectrum of mitoxantrone was very similar to that of the anthracyclines, except that the range of IC_{50} values was wider. The K562 line was clearly the most resistant haematopoietic cell line, and indeed was as resistant as the colon carcinoma cells.

Amsacrine, two of its derivatives, and an experimental acridine carboxamide [8] were most cytotoxic to the T-cell leukaemia lines (Table 2). However, as with the other intercalating agents, cell lines became progressively more refractory in the order Burkitt's lymphomas, U937, HL-60, K562 and colon lines. Thus for all classes of intercalating agents studied the patterns of relative sensitivities of the 12 cell lines were similar. These trends in intrinsic cellular responsiveness to intercalating agents were not evident when cytotoxic agents of other classes were studied. Examples of antimetabolites (5-fluorouracil, methotrexate) and alkylating compounds (nitracrine) are shown in Table 2.

DISCUSSION

The MTT assay

This report describes the use of a metabolisable dye, MTT, for rapid and reproducible colorimetric determination of the drug sensitivity of haematopoietic cells. IC_{50} values determined by this means are in good agreement with those obtained by cell counting, but show a consistent trend to slightly higher (no greater than two-fold) values (Figs. 4 and 5).

One possible reason for the higher IC_{50} values is that control (non drug-treated) cells which have grown to high densities approaching saturation are able to metabolise MTT less efficiently than cells which have been reduced to lower densities by cytotoxic compounds. MTT is reductively metabolised within mitochondria [7] and it has been reported that another compound which is accumulated by mitochondria, (rhodamine 123) is taken up less efficiently by cells at plateau phase of growth [9]. Alternatively, cell enlargement associated with drug-induced accumulation in the G2 phase of the cell cycle [10] may contribute towards the slightly higher IC_{50} values.

The metabolism of MTT is markedly nonlinear with time. This is a feature of both control and drug-treated cultures which are at reduced cell density (Fig. 3). Because the decline in MTT metabolism with time occurs independently of cell density, the IC_{50} value varies little with duration of the assay (Fig. 4).

The MTT assay is not applicable to HCT-8 cells, in which high density control cultures metabolize MTT more slowly than cultures exposed to drug at concentrations approximating the IC_{50} (data not shown). Under microscopic examination, the giant cells which survive under highly toxic drug concentrations can be seen to generate abundant crystals of product resulting in a colour reaction similar to that of control cells. Furthermore, with adherent colon carcinoma cells, the crystals of product are more difficult to solubilize as they are deposited within cells attached to the surface of the culture dish. Thus the MTT assay may not be suitable for routine use with adherent cells.

Quantitation of growth by methylene blue binding is the preferred method [5] although both assays give comparable results for SW480 and SW620 cells (Fig. 6).

Reproducibility between assays is comparable with previous methods [2, 5]. However, in earlier work, daunorubicin appeared to be less potent than doxorubicin [2, 3], whereas in this study, it is the more potent (Table 2, Fig. 7). The reason for this discrepancy is not known as the batch of daunorubicin used previously is no longer available for comparison. However, current values are in accord with the relative potencies of these two anthracyclines published by Conter and Beck [11] for CCRF-CEM cells.

Comparison of drug cytotoxicity against different cell lines

Wilson *et al.* [12] have shown that a transformed Chinese hamster fibroblast line (V79 cells) and the murine leukaemia L1210 vary markedly in their relative sensitivities to intercalating drugs. Clinically used agents were generally more active against L1210 cells, but a small number of derivatives of amsacrine were identified which were apparently more cytotoxic towards V79 cells [12]. When this study was expanded into a range of human solid tumour cell lines and a representative human leukaemia cell line, a similar range of cell sensitivities was observed [2]. However, very few compounds appeared to be selectively toxic for carcinoma (relative to leukaemia) cells in this all-human panel, and such selective cytotoxicity was observed only in two breast carcinoma lines.

The work described in the present paper, using a panel of cells derived from haematopoietic and colon malignancies, indicates that methotrexate, 5-fluorouracil, and nitracrine are in general cytotoxic for leukaemia and carcinoma cell lines at similar concentrations. However, the other drugs, which are probably all topoisomerase II poisons [15] are relatively less active against colon carcinoma cells. This trend is illustrated by the ratios of IC_{50} values for HCT-8 and Jurkat cells: for methotrexate, 5-fluorouracil and nitracrine, these are 1.9, 0.82 and 1.1 respectively. However, the

ratios for doxorubicin, mitoxantrone and amsacrine [14, 46, 15] indicate that the haematopoietic cells are intrinsically more sensitive to this broad class of agent. Recently, however, we have identified intercalating agents in which the ratio approaches 2.0 (data not shown). Whether such agents have improved therapeutic efficacy remains to be determined.

Basis of difference in sensitivities of cell lines to intercalating agents

The basis for the differences in sensitivity of cell lines towards intercalating agents has not yet been fully elucidated, but does not appear to be related to cell cycle time. Our earlier work showed there was no simple relationship between cell growth rate and sensitivity [2]. Moreover, lines both sensitive (CCRF-CEM, MOLT-4) and resistant (K562) to intercalating drugs grew at the same rate (doubling times of 24 hr during exponential growth). Two of the most rapidly growing cell lines, Jurkat and HCT-8, have doubling times of 17–19 and 15 hr respectively and represent extremes of drug sensitivity.

Clonogenicity assays (i.e. the assessment of cells for clonogenicity in semi-solid medium following a 1-hr exposure to amsacrine) have been performed for several of the haematopoietic cell lines. Results indicate that K562 cells are clearly more resistant to amsacrine than are Jurkat, MOLT-4, and U937 cells (data not shown). The lack of resistance of U937 cells is unexpected, but these results show that K562 cells are innately resistant and confirm the findings of others [6] that there is a close relationship between clonogenicity and continuous exposure assays. Differences in intrinsic activity of DNA intercalating drugs with different cell lines may arise from a variety of mechanisms. The carcinoma lines and more resistant haematopoietic lines may show increased drug efflux and/or decreased permeability to the drugs [14]. It is also possible that the degree of DNA damage caused by a given intracellular concentration of drug could vary between cell lines. The enzyme DNA topoisomerase II, which is thought to be the target molecule for amsacrine and a number of other cytotoxic drugs [15], could vary in amount or activity in different cell lines.

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