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Induction of Resistance to 6-Thioguanine and Cytarabine by a Range of Anticancer Drugs in Chinese Hamster AA8 Cells

Lynnette R. Ferguson, Christine L. Hill and Philip Morecombe

A mutagenicity assay using AA8 Chinese hamster cells has been used to explore the potential of some currently used clinical anticancer drugs to induce cells resistant to 6-thioguanine and cytarabine. Preliminary experiments gave evidence of a "low dose" and "high dose" resistance to cytarabine, and subsequent work considered only the latter of these events. When ethyl methane sulphonate was used as a reference mutagen, induced resistance to cytarabine developed substantially later and at a lower frequency than resistance to 6-thioguanine. Of the clinical drugs tested, carmustine showed the highest ability to induce either 6-thioguanine or cytarabine resistant cells. Bleomycin, daunomycin and amsacrine showed moderate ability, while vincristine was essentially inactive in these assays. Such information could potentially be used in selecting new drug combinations or timing of drug administration in cancer chemotherapy.
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INTRODUCTION

A MAJOR limitation to successful chemotherapy for cancer is the development of resistance to agents used in its treatment. There are often some drug resistant cells originally present in the tumour, and these will increase in frequency as the sensitive cells are selectively killed [1]. Additionally, mutagenic drugs (which are commonly used in cancer chemotherapy), may mutate the cells which survive treatment, thereby enhancing resistance to other agents in subsequent cycles of a chemotherapeutic schedule.

In selecting drug combinations for chemotherapy, it is clearly relevent to ask whether the drugs selected will induce resistance to other selected drugs. Although there are a number of mutagenicity studies on anticancer drugs available in the literature, many of these have utilised a variety of endpoints which are not relevant to cancer chemotherapy. For example, two drugs commonly used in antileukaemic schedules are 6-thioguanine and cytarabine. These have been combined with daunomycin, and more recently with amsacrine in antileukaemic therapy [2]. Although both daunomycin and amsacrine have been shown to induce resistance to 6-thioguanine [3], there have been no studies to show whether they induce resistance to cytarabine.

Resistance to 6-thioguanine is a common endpoint in mammalian mutagenesis studies, and optimal conditions to detect this have been determined for a variety of mutagens in a range of cell lines including Chinese hamster V79 [3] and CHO cells [4], and mouse lymphoma L5178Y [5]. Although Cole *et al.* [5] and Rogers *et al.* [6] estimated resistance to cytarabine, the mouse lymphoma cell line used shows both a low spontaneous and also a low induced incidence of this, and there were practical difficulties in studying sufficient cells for statistical significance. The spontaneous incidence of cytarabine resistance in Chinese hamster cells has been recorded as around 1×10^{-5}, a cell number which is more practical for laboratory studies [7].

Correspondence to L.R. Ferguson.
The authors are at the Cancer Research Laboratory, University of Auckland Medical School, Private Bag, Auckland, New Zealand.
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In this study, we have determined the conditions for studying induction of resistance to 6-TG and to cytarabine by a reference alkylating agent (ethyl methanesulphonate, EMS) in AA8 Chinese hamster cells. Micronuclei have been scored in the same experiments, in order to provide an index of clastogenic activity. Using the optimised system, we have then proceeded to study induction of both types of drug resistance by amsacrine, bleomycin, carmustine, daunomycin and vincristine, as examples of clinically used antineoplastic drugs with differing modes of action. Both amsacrine and daunomycin appear to break DNA through inhibition of the DNA-associated enzyme, topoisomerase II. Bleomycin is thought to act directly as a DNA cutting agent. Carmustine is known as a bis-functional alkylating agent, while vincristine acts on the mitotic spindle.

MATERIALS AND METHODS

Drugs

EMS was purchased from Sigma; 6-thioguanine from Aldrich; bleomycin and carmustine from Bristol-Myers; cytarabine and vincristine from David Bull Laboratories Ltd., (Victoria, Australia) and daunomycin from Warner Lambert. The isothionate salt of amsacrine was kindly provided by Dr W.A. Denny, (Auckland Division, Cancer Society of New Zealand). A sterile stock solution of 6-thioguanine (1 mg/ml in Na_2CO_3) was stored at −20°C. Fresh solutions of all other drugs were prepared immediately before use. All of the compounds, with the exception of bleomycin, were pure as estimated by thin layer chromatography. Bleomycin was a mixture of different forms, although the predominent form was bleomycin A2. In calculating the molarity of bleomycin solutions we have assumed an average molecular weight of 1514.

Cells

Chinese hamster AA8 cells were obtained from Dr W.R. Wilson in this laboratory. The line was maintained in suspension culture in α-minimal essential medium (α-MEM) without nucleosides or antibiotics, containing 10% (v/v) heat-inactivated fetal calf serum (Gibco, New Zealand) and subcultured to 10^4

cells/ml twice weekly. The line was free of mycoplasma as judged by cytochemical staining [8].

Low spontaneous levels of resistance to both 6-thioguanine and cytarabine were maintained by reducing cell numbers to 1×10^3 cells once a week, thereby eliminating revertant clones by limiting dilution [3].

Drug exposure and determination of cell killing

10 ml of exponential-phase cultures initiated 24 h previously at 1.5×10^5 cells/ml [in α-MEM containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml)] were transferred to McCartney bottles. Drug was added in no more than 50 μl of 50% ethanol, and the cultures incubated in the CO_2 incubator for 1 h at 37°C with bottles being inverted every 10–15 mins. A typical experiment included two solvent controls and five drug concentrations, chosen on the basis of a preliminary toxicity experiment. Drug treatment was terminated by transferring cells to a centrifuge tube, spinning and washing cells three times with phosphate buffered saline (NaCl, 8g/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). Cell densities were determined with an electronic particle counter (Coulter Electronics). Cell survival was assessed by plating up to 10^4 cells in 5 ml growth medium in 60 mm tissue culture petri dishes, growing for 8 days in standard conditions. 8 days later, plates were washed with phosphate buffered saline (PBS) and colonies stained with 0.5% methylene blue in 50% alcohol for 10 min. Colonies were counted manually and the surviving fraction of cells was determined relative to the plating efficiency of the controls, which was in the range 60–90%.

Expression and selection of mutants

After drug treatment, cells were subcultured in growth medium to give between 1 and 4×10^6 clonogenic cells per 100 mm petri dish, estimated on the basis of previous survival curve determinations. Cells were subsequently maintained in exponential-phase growth by subculturing to 10^6 cells/100 mm dish every 2 days to allow expression of the mutant phenotype. After the stated expression time, mutants were selected as described in the individual sections. At the same time, 100 mm petri dishes containing 15 ml of growth medium were seeded with 120 cells (in triplicate for each culture) to determine the plating efficiency at the time of mutant selection. Selection dishes were incubated for 11 days while non-selective dishes were incubated for (usually) 8 days, to allow colony formation. The mutation frequency was calculated as the ratio of the plating efficiency in selective media to that under non-selective conditions. The errors shown for the mutation frequency are root mean square averaged standard errors based on colony counts in replicate selective and non-selective plates.

The significance of the data was tested by linear regression analysis of trends in the mutation frequency with drug concentration [3]. Significance of individual values of the correlation coefficient, r, were determined using tables derived from Fisher's normalising arc-tanh transformation with n-2 d.f. Using this test, agents were classified as mutagenic if the gradient of the regression line was significantly greater than zero at $P = 0.05$.

Determination of micronuclei

Drug-induced chromosome breakage was assessed by counting micronuclei in Giemsa stained cells prepared 2 days after drug treatment. Trypsinised single-cell suspensions were fixed in ice-cold Carnoy's fixative (3:1 v/v methanol:acetic acid) after swelling in hypotonic KCl (0.075 mol/l) for 6 min at 37°C, and were dropped 20 cm onto clean glass slides. Cytoplasmic structures were scored as micronuclei if they showed the same Giemsa staining reaction as the nucleus, were clearly resolved from the nucleus, and had diameters in the range 2.5–10 μm. The range of nuclear diameters in these slides was 12.5–27.5 μm for control cells. Either 100 cells with micronuclei or 2000 cells in total were scored for each data point.

Cloning of drug resistant cells

20 of each type of resistant clones arising spontaneously were dispersed by adding trypsin to cells within a cloning ring and removing the released cells to a 60 mm dish with fresh growth medium plus 10% fetal calf serum. On reaching confluence, the cells were trypsinised, resuspended, counted and diluted to give one cell per 0.1 ml growth medium. This amount was placed into each of 18 wells [within a 96 well microtitre tray (Nunc)]. Each clone arising within a well was assumed to have come from a single original cell, and one such clone was further expanded in cell number by replating into a 60 mm dish with fresh medium. When these cells were confluent they were tested for ability to survive treatment with the appropriate drug (6-thioguanine or cytarabine).

RESULTS

Inhibition of colony forming ability

In preliminary experiments and in line with results from other workers [3–5], we found that the survival of cells plated in the presence of 6-thioguanine decreased exponentially up to a concentration of 2–2.5 μg/ml, and survival plateaued after this point (due to the presence of 6-thioguanine resistant cells). In the final assays, cells were plated at a density of 2×10^5 per plate and 6-thioguanine resistant colonies were selected in a medium containing 3% fetal calf serum with 5 μg/ml 6-thioguanine. When resistant 6-thioguanine clones were grown and retreated with a dose range of 6-thioguanine, 20/20 tested retained resistance to 6-thioguanine.

Increasing concentrations of cytarabine also led to an exponential decrease in cell survival up to concentrations around 0.5 μmol/l. Beyond this point survival plateaued, but the exact concentration at which this occurred depended on the number of cells plated. For cells plated at concentrations of 2×10^5/plate or less, a low dose resistance was seen, plateauing around 0.83 μmol cytarabine. When higher cell concentrations (1×10^6 cells/dish) were plated, then a "high dose" resistance occurred, with a plateau around 3 μmol/l. The effects of varying cell numbers on the number of cells surviving at 3 μmol/l cytarabine is illustrated in Fig. 1. In practice, it was our experience that variable results were obtained if drug resistant cells were selected at the lower drug concentration. When clones resistant to low dose cytarabine were grown and retested with cytarabine, only 9/20 retained resistance to the 0.83 μmol/l concentration. A restruction experiment (data not presented) confirmed that there was a good recovery of cytarabine resistant clones from plates selected under the "high dose" conditions. When clones resistant to high doses cytarabine were grown and retested with a dose range of cytarabine, all 20/20 retained resistance to 3 μmol/l cytarabine. Therefore, in subsequent work we selected cytarabine resistant clones by plating 1×10^6 cells/dish, in the presence of 3 μmol/l cytarabine. We used the same batch of fetal calf serum for all experiments, as preliminary experiments suggested that the batch also affected the recovery of mutant clones (data not presented).

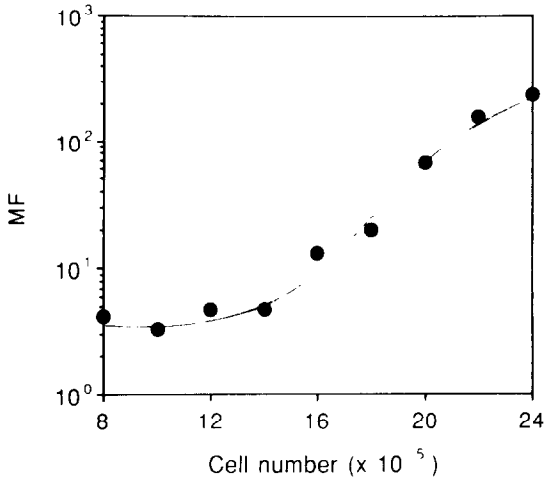


Fig. 1. The effects of plating various cell numbers/dish on the apparent cloning efficiency of AA8 cells in the presence of 3 μmol/l cytarabine.

Time to formation of micronuclei, 6-thioguanine and cytarabine resistant clones after EMS treatment

EMS is known to increase the frequency of cytarabine resistant cells [7, 9, 10]. Therefore, although EMS in not an anticancer drug, it was utilised as a standard in preliminary experiments, in order to optimise treatment and selection procedures. AA8 cells were treated with a range of EMS concentrations and either plated for subsequent micronuclei measurements or expression of drug resistance, or diluted so that survival could be estimated. Expression plates were harvested at various intervals afterwards for estimation of micronuclei (Fig. 2) as well as 6-thioguanine and cytarabine resistant cells (Fig. 3). Each of these parameters was significantly increased by the EMS treatment, and the optimal expression time for detection varied considerably. Although 2 or 3 days appeared to be the optimal time after treatment for estimating micronuclei levels, 6-thioguanine resistance reached a maximum at 4–6 days, while cytarabine resistance required 8 days or more for optimal expression of mutation frequency.

For subsequent work, a standard expression time of 2 days was used for micronuclei, 4 and 6 days expression for 6-thioguanine resistance and 8 and 10 days for cytarabine resistance. The effects of increasing dose on survival, micronuclei induction and induction to 6-thioguanine and cytarabine resistance by EMS are illustrated on Fig. 4.

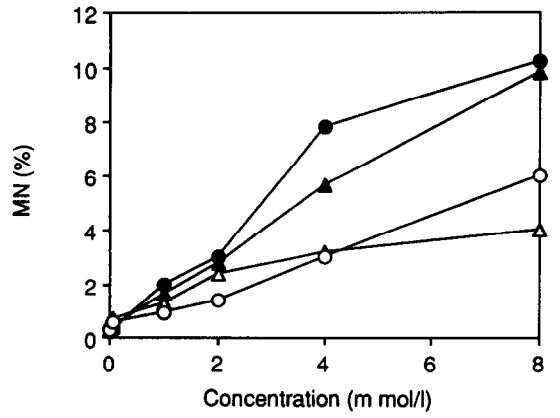
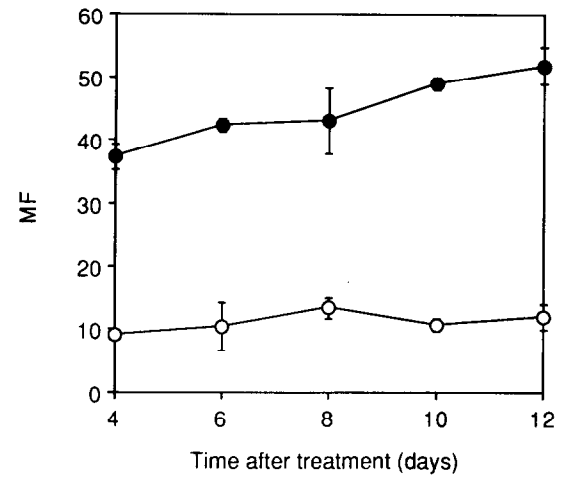


Fig. 2. Micronuclei frequencies at various times after treatment of AA8 cells for 1 h with three different EMS concentrations. ○ 1 day; ● 2 days; ▲ 3 days; △ 4 days.



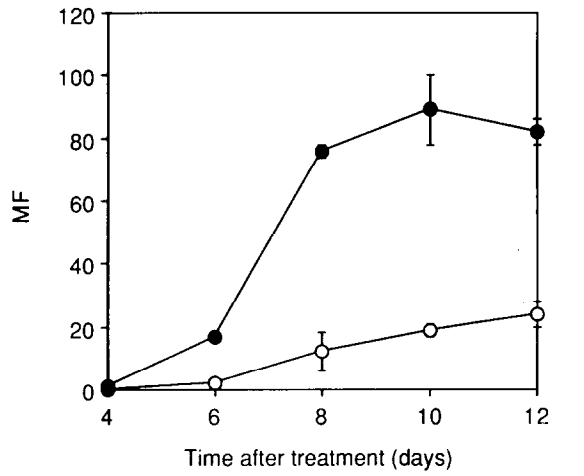


Fig. 3. Dependence of mutation frequency × 10^5 on expression time following 2 (○) or 8 (○)mmol/l of EMS for 1 h. Expression plates were harvested at various time intervals after drug treatment for estimation of 6-thioguanine (upper panel) and cytarabine resistant clones (lower panel).

Induction of micronuclei, 6-thioguanine and cytarabine resistance by clinical anticancer drugs

The antitumour agents were all tested in the system developed above, and a summary of these data are compared with those for EMS in Tables 1 and 2. Although five agents were tested, the data for the clinical agents showed essentially only three different patterns, illustrated on Figs 5–7. Carmustine (Fig. 5) was moderately active in micronuclei induction, an effective inducer of 6-thioguanine resistance and moderate inducer of cytarabine resistance. For example, in the first experiment, spontaneous levels of 6-thioguanine resistance were 4.7×10^{-6}, but after 50 μmol/l carmustine survival was reduced to 9.4%, while the frequency of 6-thioguanine resistant cells among those surviving the drug treatment was estimated at 236×10^{-6} after 4 days expression time, 211×10^{-6} after 6 days expression time. All experiments on induction of 6-thioguanine resistance reached high levels of statistical significance (Table 2). By contrast, the spontaneous level of cytarabine resistance in the same experiment 8 days after 1 h treatment with 50 μmol/l carmustine was 1.7×10^{-6}, the frequency of cytarabine resistant clones

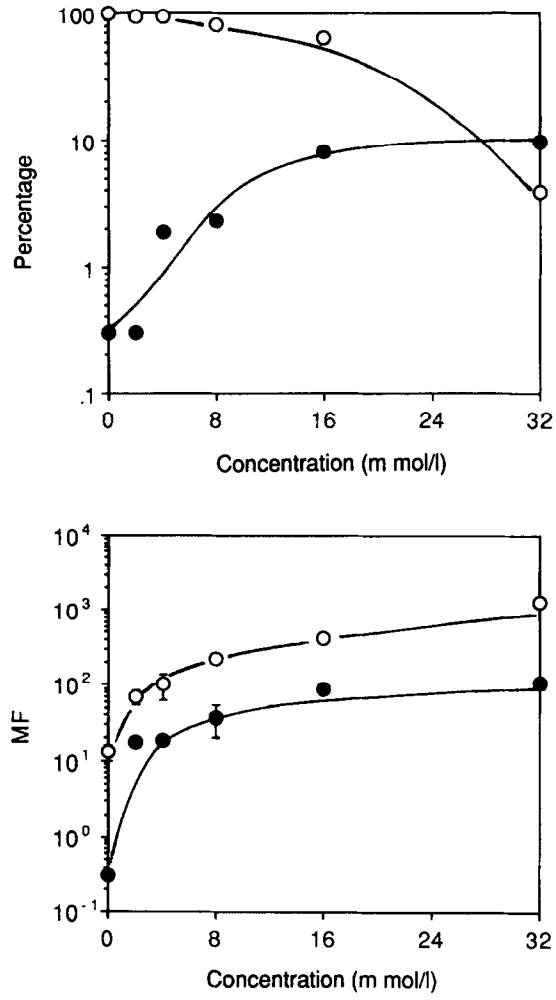


Fig. 4. The effects of increasing dose of EMS for 1 h on survival and micronuclei induction (upper panel) and MF (lower panel). The surviving fraction (○) was determined by diluting cells into fresh growth medium at the end of the drug treatment period, and the percentage of cells with micronuclei was determined 2 days later. (In the right hand panel, mutation frequency) is expressed with respect to surviving cells at the time of selection. ○ 6-thioguanine resistant mutants, selected after 4 days expression; ● cytarabine resistant clones, selected after 8 days expression.

reached 11.4 × 10^{-6}, and after 10 days this reached 8.4 × 10^{-6}. Only three out of four sets of data reached statistical significance.

The three drugs amsacrine, daunomycin and bleomycin were very effective clastogens as estimated by micronucleus levels. In terms of molar potency, the first two of these drugs were significantly more effective at inducing 6-thioguanine and cytarabine resistant cells than either of the two alkyating agents. However, this is partly an artifact caused by the considerable dose potency of the two drugs. If we consider a measure of either clastogenesis or drug resistant cells induced as a function of cell survival (e.g. the number of mutants induced at the D_{37}) then amsacrine, daunomycin and bleomycin are revealed as moderate inducers of 6-thioguanine resistance but only weak inducers of cytarabine resistance. The data for bleomycin resistance was 2.7 × 10^{-6}, of cytarabine resistance was 1.3 × 10^{-6}. Following treatment with 130 μ mol/l bleomycin, survival was reduced to 2.7%, while the estimated frequency of 6-thioguanine resistant cells rose to 81 × 10^{-6} after 4 days expression time, 92 × 10^{-6} after 6 days. In the same experiment, cytarabine

Table 1. Cytotoxicity and clastogenesis following 1 h drug exposure

Drug	Exp. No.	Cytotoxicity D_{37} (μmol/l)*	Clastogenesis: Molar potency†	Clastogenesis: Activity ‡ at D_{37}
EMS	1	21600	0.0003	7.5
	2	18400	0.0003	6.3
Carmustine	1	36	0.10	4.3
	2	33	0.12	3.9
Amsacrine	1	0.6	8.0	4.8
	2	0.7	7.4	5.2
Daunorubicin	1	0.9	6.8	6.1
	2	0.9	6.9	6.2
Bleomycin	1	8	57	4.3
	2	11	55	5.9
Vincristine	1	98	0.02	2.0
	2	103	0.02	2.3

*D_{37} = μmol/l concentration which reduces AA8 cell survival to 37%.
†Molar potency is the gradient of the linear regression curve relating the percentage of cells with micronuclei to drug concentration. The correlation was statistically significant ($P < 0.05$) in all cases.
‡The interpolated percentage of cells with micronuclei at D_{37} after subtraction of control frequencies.

resistant cells reached a frequency of 5.6 × 10^{-6} after 8 days expression, 7.6 × 10^{-6} after 10 days. Although each of the four sets of data on 6-thioguanine resistance reached statistical significance, one of the cytarabine resistance data sets failed to reach significance at the 5% level.

Daunomycin is a potent anticancer drug, killing 90% of cells at a concentration of 2 μmol/l. In our first experiment with this drug, treatment of cells with a 2 μmol/l concentration led to 7.9% of cells with micronuclei. A spontaneous level of 3.6 × 10^{-6} 6-thioguanine resistant cells rose to 76 × 10^{-6} after 4 days and 104 × 10^{-6} after 6 days expression time. Cytarabine resistant cells were initially at a frequency of 2.5 × 10^{-6}, rising to 5 × 10^{-6} after 8 days and 13.9 × 10^{-6} after 10 days expression time. Three out of four sets of data reached statistical significance for both 6-thioguanine and cytarabine resistance. The initial experiment with amsacrine gave very similar increases in 6-thioguanine resistance to those by daunomycin (from 5.3 to 99.7 or 107 × 10^{-6} frequency of mutant clones), and all values obtained were statistically significant (Table 2). However, the data for cytarabine resistance failed to reach statistical significance (Table 2) despite increasing at least 2-fold in each set of data (e.g. from 0.7 to 2.4 or 1.4 × 10^{-6}).

In contrast with the other agents tested, vincristine (Fig. 7) was less active in causing micronuclei, and those which occurred were generally larger than those seen for the other drugs. Additionally, there was no evidence whatsoever for induction of 6-thioguanine or cytarabine resistance by this anticancer drug. If anything, the spontaneous values showed a decline, although this was not statistically significant (Table 2).

DISCUSSION

Our first objective in these studies was to develop an assay system which could be used in order to compare anticancer drugs in their ability to induce resistance with two others which might be selected for combination chemotherapy. It is important that such assays be reproducible and also that the drug resistances seen relate to those in the clinic.

Table 2. Mutagenesis following 1 h drug exposure

		Mutagenesis									
		6-Thioguanine resistance					Cytarabine resistance				
Drug	Exp. No.	Exp.*	Molar† Potency	r‡	P§	Act.‖ at D_{37}	Exp.	Molar potency	r	P	Act. at D_{37}
EMS	1	4	0.035	0.988	≤0.001	755	8	0.004	0.908	≤0.05	87.5
		6	0.050	0.985	≤0.001	1073	10	0.005	0.919	≤0.01	97.6
	2	4	0.062	0.999	≤0.001	1147	8	0.005	0.917	≤0.01	90.0
		6	0.051	0.966	≤0.01	943	10	0.006	0.895	≤0.05	116.6
Carmustine	1	4	3.65	0.973	≤0.01	131	8	0.178	0.961	≤0.01	6.4
		6	4.31	0.928	≤0.01	155	10	0.127	0.738	N.S.¶	4.6
	2	4	1.92	0.995	≤0.001	63	8	0.343	0.853	≤0.05	11.3
		6	2.13	0.927	≤0.01	70	10	0.498	0.964	≤0.01	16.4
Amsacrine	1	4	45.9	0.909	≤0.05	28	8	3.31	0.754	N.S.	2.0
		6	51.5	0.996	≤0.001	32	10	3.87	0.802	N.S.	2.3
	2	4	62.1	0.993	≤0.001	43	8	2.46	0.517	N.S.	1.7
		6	54.3	0.997	≤0.001	38	10	1.43	0.436	N.S.	1.0
Daunorubicin	1	4	42.6	0.836	≤0.5	38	8	1.67	0.796	N.S.	1.5
		6	35.4	0.455	N.S.	32	10	2.23	0.853	≤0.05	2.0
	2	4	28.6	0.948	≤0.001	26	8	5.34	0.886	≤0.05	4.8
		6	25.8	0.976	≤0.001	23	10	5.03	0.921	0.01	4.5
Bleomycin	1	4	0.628	0.983	≤0.001	28	8	0.374	0.952	≤0.01	2.9
		6	0.622	0.950	≤0.01	32	10	0.719	0.767	≤0.01	4.3
	2	4	0.435	0.921	≤0.01	24	8	0.432	0.986	N.S.	4.7
		6	0.446	0.934	≤0.01	20	10	0.746	0.990	≤0.001	8.0
Vincristine	1	4	−0.001	0.034	N.S.	—	8	−0.001	0.131	N.S.	—
		6	0.002	0.026	N.S.	—	10	0.001	0.387	N.S.	—
	2	4	−0.03	0.416	N.S.	—	8	−0.002	0.293	N.S.	—
		6	0.02	0.380	N.S.	—	10	0.001	0.351	N.S.	—

*Expression time in days.
†The gradient of the linear regression curve relating the mutation frequency (as mutants/10^6 clonogenic cells) to μmol/l drug concentration.
‡r = Correlation coefficient for the linear regression.
§P = Probability that the mutagenic activity is not significant.
‖The interpolated mutation frequency at the D_{37} after subtraction of the control frequency.
¶N.S. = not significant.

Resistance to 6-thioguanine can be easily studied as a mutagenesis endpoint. Although there is value in checking the exact conditions appropriate to a given cell lines, the system allows some flexibility in cell numbers which can be plated, and a wide range of serum batches could be used. There is a large literature on development of 6-thioguanine resistance in the clinical situation, as well as a large body of experimental mutagenesis data. In either case, resistance primarily arises from defects at the hypoxanthine guanine phosphoribosyltransferase locus, an event which can be caused by mutation at the gene or chromosomal level [11, 12].

Resistance to cytarabine has been less extensively considered as a mutagenesis assay, and the present work has confirmed that selection for cytarabine is less easily studied than selection for 6-thioguanine. Mutant selection requires precise attention to cell numbers, and is also affected by serum batch. Comparing our own selection conditions with those utilised by others [5, 6] suggests that if the assay were to be taken up by other laboratories, then it would be essential to perform the same preliminary experiments for a given cell line and fetal calf serum batch, rather than simply utilising our conditions.

de Saint Vincent and Buttin [7] showed that there were two main types of cytarabine resistant variants isolated from mutagenised Chinese hamster fibroblasts. Those with a high level of resistance were mostly deoxycytidine kinase deficient, while those with resistance to a lower cytarabine concentration showed an increased pool of deoxycytidine triphosphate. Tattersall *et al.* [13] and Kees *et al.* [14] demonstrated that this mechanism (deoxycytidine kinase deficiency) is one of the major mechanisms of resistance in blast cells of leukaemia patients clinically resistant to cytarabine. Increased cytidine deaminase activity has also been demonstrated in clinical samples by Steuart and Burke [15]. Defective membrane nucleoside binding sites, leading to defective transport also appear to be involved in cytarabine resistance in acute human leukemia [16].

There have also been previous studies on mutation to cytarabine resistance by various agents. Harris [17] induced cytarabine resistance in Chinese hamster cells after treatment with nitrosoguanidine, followed by a 6 day expression time, but did not develop this further as a mammalian mutagenesis assay. Cole and Arlett [5] demonstrated resistance to cytarabine in L5178Y mouse lymphoma cells, and Rogers *et al.* [6] used these cells to study mutation to cytarabine resistance. Variants resistant to 1 μmol/l cytarabine were induced in a dose depen-

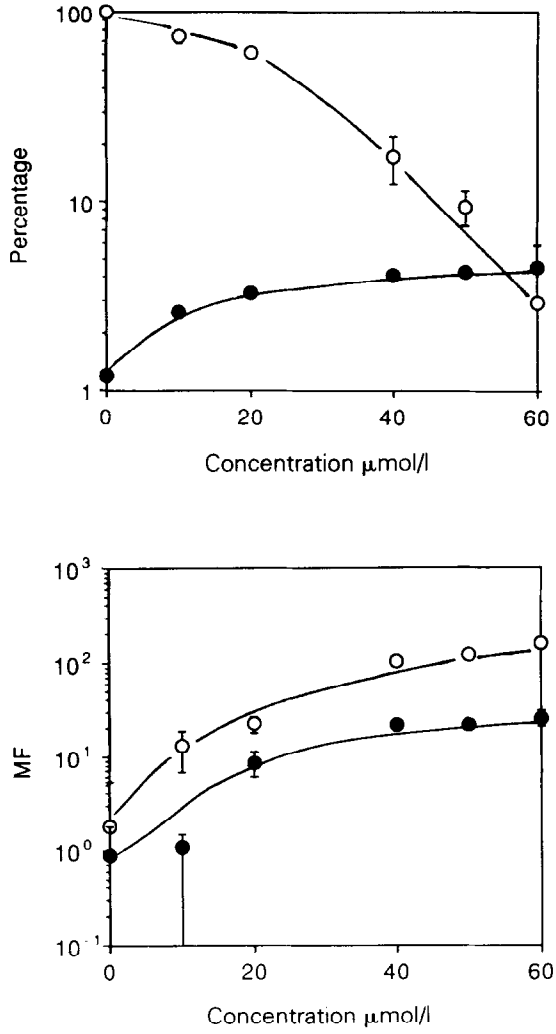


Fig. 5. The effects of increasing dose of carmustine for 1 h on survival and micronuclei induction (upper panel) and mutation frequency (lower panel). Symbols as for Fig. 5.

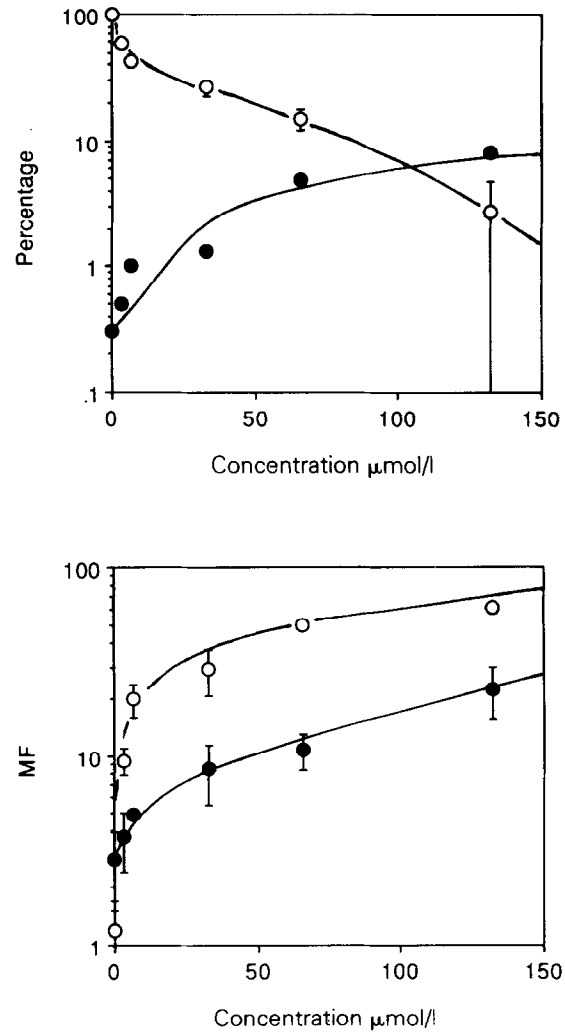


Fig. 6. The effects of increasing dose of bleomycin for 1 h on survival and micronuclei induction (upper panel) and mutation frequency (lower panel). Symbols as for Fig. 5.

dent fashion following treatment with EMS, ethidium bromide, 2-acetylaminofluorene, but not with gamma irradiation. However, the spontaneous frequency was around 3×10^{-8}, meaning that practical studies required growth of very large numbers of cells while statistical evaluation of the data was clearly difficult. Nevertheless, the increase in cytarabine resistant cells after EMS treatment in their studies was very comparable to the increase seen in the present study.

Both spontaneous and inducted resistance to 6-thioguanine occurs at much higher levels than for cytarabine in the AA8 Chinese hamster line. The HGPRT locus is on the X-chromosome, and the AA8 cell line is XY. This means that the locus can be inactivated by a single event, and this could involve a mutation at either the gene or chromosome level. However, resistance to cytarabine may be caused by mutations at any of the several different loci, and a variety of events could be associated with this including gene or chromosomal mutation, mitotic crossing-over or even gene amplification [18]. It is interesting to note that Chinese hamster cell lines appear to show a high degree of heterozygosity and this may relate to the higher cytarabine resistance in these as compared with other cells [7].

The induction of chromosomal mutations was implied in these studies by estimating micronuclei. For EMS, carmustine, bleomycin, daunomycin and also for amsacrine, the level of micronuclei at the D_{37} was almost identical. These data would be compatable with chromosome breakage being causal in cell death. However, there is no direct relationship between 6-thioguanine or cytarabine resistance with micronuclei for each of these drugs. Although chromosomal effects could be responsible for some of these resistant cells, it is clearly not the only mechanism. Vincristine did induce micronuclei, but levels were lower than for the other agents. These micronuclei may well have resulted from the loss of whole chromosomes rather than from chromosome breakage [19], leading to different relationships between micronuclei and cell death as compared with the other drugs. As well as inducing drug resistance, one of the other drawbacks in current clinical drugs is that they may induce second, therapy-related cancer [20]. These effects may also be related to their mutagenic properties. Results obtained from mutagenicity assays such as these may also predict relative activity of the drugs as carcinogenic agents, and could be used to select agents with low carcinogenic potential.

The most important aim of the present study was to understand whether current clinical agents do induce resistance to other agents within a clinical schedule. The studies have shown

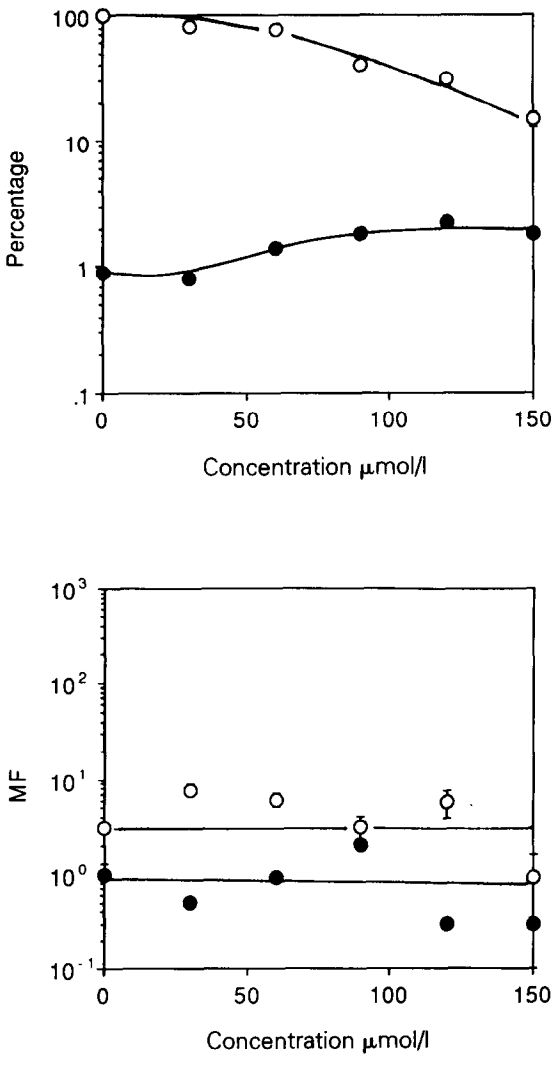


Fig. 7. The effects of increasing dose of vincristine for 1 h on survival and micronuclei induction (upper panel) and mutation frequency (lower panel). Symbols as for Fig. 5.

that there are significant differences between agents in inducing resistance to both 6-thioguanine and cytarabine. There are also significant differences in times at which different types of resistance emerge. It is clear that alkylating agents are significantly worse than drugs which cause chromosome breakage through other mechanisms. Additionally, the mitotic spindle inhibitor, vincristine was significantly better than any of the other agents used. It is tempting to speculate that the success of daunomycin and also amsacrine in antileukaemic schedules with cytarabine and 6-thioguanine [2] is that they are not particularly effective in inducing resistance to the other two drugs. We suggest that assays such as the present, particularly if they are extended to consider resistance to other drugs, could provide ancillary information which could be of value in developing new protocols and more rational drug combinations for anticancer therapy.

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