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Mutagenicity Profiles of Newer Amsacrine Analogues with Activity against Solid Tumours: Comparison of Microbial and Mammalian Systems

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Abstract—*Amsacrine, an acridine derivative used clinically in the treatment of acute leukaemia, has formed the basis for the development of further compounds with high activity against experimental solid tumours, one of which is currently in clinical trial. We have compared the ability of these drugs to cause point mutations in bacteria, 'petite' mutations in yeast and mutations in mammalian cells. Several of the compounds are frameshift mutagens in* Salmonella typhimurium *TA*1537 *while some cause 'petite' mutagenesis in* Saccharomyces cerevisiae. *All are highly clastogenic and have significant mutagenic activity at the* 6-*thioguanine locus in cultured V*79 *Chinese hamster fibroblasts following* 1 h *drug exposures. None are mutagenic at the ouabain locus of these cells. The relationship between different indicators of mutagenicity has been studied using an additional set of amsacrine analogues, some of which are mutagenic in* S. typhimurium *TA*98. *There is a highly significant relationship between mutation frequency (measured as resistance to* 6-*thioguanine) and either cytotoxicity (D_{37} values in a clonogenic assay) or clastogenicity (ability to induce micronuclei). However, there is no correlation with mutagenicity in microbial systems. The results suggest that the cytotoxicity, clastogenicity and mutagenic activity of the amsacrine analogues is mediated by similar mechanisms, probably involving the enzyme DNA topoisomerase II.*

INTRODUCTION

AMSACRINE [1] is a derivative of 4′-(9-acridinylamino)methanesulphonanilide (see Fig. 1 for structures) used clinically for treatment of leukaemia [2]. Amsacrine is mutagenic to microbial cells [3–5] and in mammalian cells it causes sister chromatid exchange [6, 7], chromosomal aberrations [8, 9], micronucleus formation, mutagenesis [10, 11] and cell transformation [12]. All of these properties suggest that, in common with many other antitumour drugs [13], amsacrine is a potential carcinogen [10]. Additionally, these properties may give it the potential to induce resistance both to itself and to other agents during chemotherapy. It would be useful to be able to design analogues which minimize such mutagenicity, and it appears that analogues can be synthesized with lowered mutagenicity at least in microbial systems [3].

Several amsacrine analogues appear to have superior antitumour activity to amsacrine in animal models [14, 15] and one of these is currently in clinical trial [16]. Several series based on further structural types (Fig. 1) have been developed and many of these also have higher experimental antitu-

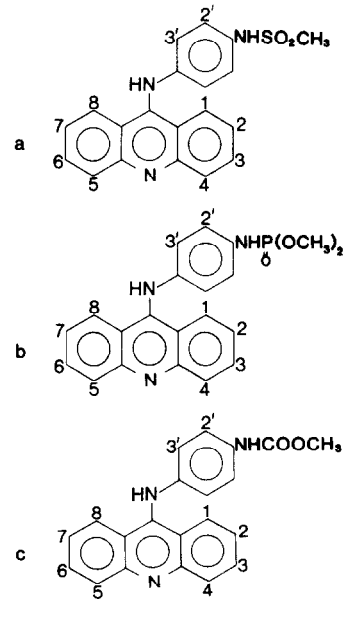

Fig. 1. Structures of amsacrine analogues referred to in the text and in Tables 1 *and* 2.

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mour activity than amsacrine [17–20]. It is therefore of interest to investigate whether these newer compounds combine high antitumour activity with low mutagenic activity, and furthermore to determine whether microbial results extend to mammalian cell mutagenicity.

In this study, we have compared the mutagenic activity of a series of derivatives of 9-anilinoacridine (Fig. 1). We have quantitated mutagenicity in bacteria using frameshift and point mutagenesis, in yeast cells using the induction of 'petite' colonies, and in mammalian cells using the induction of 6-thioguanine and ouabain resistance in V79 Chinese hamster cells. The clastogenic potential of the drugs has also been estimated in the same experiments by scoring micronuclei in interphase cells. Drugs have been selected in order to cover a spectrum of patterns of microbial mutagenicity as well as agents with high activity against experimental solid tumours.

METHODS AND MATERIALS

Chemicals

9-Anilinoacridine derivatives were synthesized in this laboratory as previously described [17–20] and were pure as judged by thin layer chromatography. 9-Aminoacridine, 4-nitro-*o*-phenylene diamine, sodium azide and ouabain were purchased from Sigma Chemical Co., U.S.A., and 6-thioguanine was from Aldrich Chemical Co., U.S.A. Sterile stock solutions of 6-thioguanine (1 mg/ml in 0.5% Na_2CO_3) were stored at −20°C. Fresh solutions of all other chemicals were prepared immediately before use.

Bacterial and yeast strains

S. typhimurium strains TA1537, TA98 and TA100 were kindly provided by Professor B.N. Ames, Department of Biochemistry, University of California, Berkeley, California. *S. cerevisiae* strain D5 [21] was kindly provided by Dr. B.S. Cox, Botany School, Oxford University, U.K. Upon receipt, all strains were grown to stationary phase in the appropriate liquid complete medium, and frozen (with 10% dimethylsulphoxide) in 1 ml aliquots at −80°C.

Mammalian cells

The Chinese hamster fibroblast line V79-171b was originally obtained from Dr. W.R. Inch, London, Ontario, and was provided by Dr. W.R. Wilson. It was maintained in α-MEM containing 10% v/v heat inactivated foetal calf serum (Gibco, New Zealand) without antibiotics and subcultured by trypsinization and dilution to 10^4 cells per 25 cm^2 flask twice weekly. In the mutagenesis assay cells were subcultured to 10^3 cells/flask 7 days prior to setting up cells for the assay in order to eliminate spontaneous 6-thioguanine mutants by limiting dilution [10]. The cell line subcultured in this manner has been designated V79-L. Cell cultures were maintained in humidified incubators with an atmosphere of 5% CO_2 in air at 37°C. The cell line was free from *Mycoplasma* as judged by cytochemical staining [22].

Bacterial mutagenicity assay

For each experiment, a vial was removed from −80°C storage, inoculated into fresh bacterial complete medium (20 ml) and grown for 4 h. To ensure that all cultures were in the same stage of growth when used, the optical density of the culture was checked at 15 min intervals until a 10-fold dilution into fresh bacterial complete medium gave an absorbance reading of between 0.11 and 0.12 at 654 nm. The *S. typhimurium* plate incorporation assay was carried out as described [23] with each experimental point performed in triplicate on at least two separate occasions. Positive controls (9-aminoacridine for TA1537, 4-nitro-*o*-phenylene diamine for TA98 and sodium azide for TA100) were included in all experiments. Mutation frequencies were calculated from the slopes of linear regression lines in the dose range where the compound showed little or no toxicity as judged by examination of the background bacterial lawn.

'Petite' mutagenesis assay

Yeast cells were exposed to a graded series of drug concentrations, using the microtitre assay previously described [24]. After growth (2 h) in the presence of drug, cells were washed by dilution and plated on each of 3 YC plates [25]. These were incubated for 3 days at 30°C before being scored for survival and for 'petite' mutagenesis.

Mammalian mutagenesis assay

Exponential phase cultures initiated 24 h previously at 1.5×10^5 cells/ml in growth medium (α-MEM containing 10% foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin; 10 ml per 100 mm Petri dish) were treated with drug (60 min) in prewarmed growth medium as previously described [10]. Cell survival was assessed by plating up to 10^4 cells in plating medium (as above but with 3% foetal calf serum; 5 ml per 60 mm Petri dish). Colonies were counted 8 days later after staining with 0.5% methylene blue in 50% ethanol. The plating efficiency of controls was in the range 60–90%. After drug treatment, cells were subcultured in growth medium to give approx. 10^6 but not more than 4×10^6 clonogenic cells per 100 mm Petri dish. Cells were subsequently maintained in exponential phase growth by subculturing to 10^6 cells every 2 days to allow expression of the mutant phenotype. After expression periods of 4 days, and in at least one experiment for each

drug at 6 days, mutants were selected by plating 6×10^5 cells in 100 mm Petri dishes (four replicates per culture) containing 15 ml of plating medium with ouabain (3 mM) or 6-thioguanine (5 μg/ml). At the same time, 100 mm dishes containing 15 ml plating medium were seeded with 120 cells (in triplicate for each culture) to determine the plating frequency at the time of mutant selection. Dishes were incubated for 11 days (selection dishes) or 8 days (non-selection dishes) to allow colony formation. The mutation frequency was calculated as the ratio of plating efficiency in selective medium to that in unselected conditions. The standard errors for mutation frequencies are based on colony counts in replicative selective and non-selective plates. Agents were classed as mutagenic if the gradient of the regression line was significantly greater than zero ($P < 0.05$) [10].

Micronucleus induction assay

Micronuclei were studied in parallel with the mutagenesis experiments, in order to correlate a measure of clastogenesis with survival and mutation. Giemsa stained cells were prepared 2 days after drug treatment for 60 min as described previously [10]. Either 100 cells with micronuclei or 2000 cells in total were scored for each data point. Estimates of the molar potency of clastogenesis were derived from the gradients of the linear regression lines giving best fit to the micronucleus induction frequency as a function of drug concentration. Drug concentrations giving survival levels of less than 20% were excluded from analysis, since there is clear evidence for non-linearity at high doses (this results from inhibition of progression of damaged cells through mitosis; Dr. W.R. Wilson, pers. comm.).

RESULTS

Selection of compounds

The compounds selected for potential clinical interest included those developed on the basis of new anilino substitution patterns (Fig. 1) and which have been submitted to the National Cancer Institute, U.S.A., for further biological evaluation (Table 1). They cover a more than 100-fold range in DNA association constants and all have activity against the Lewis lung carcinoma in mice.

Other compounds, which were selected on the basis of microbial mutagenicity profiles, are presented in Table 2. None are highly active against the Lewis lung carcinoma. Three are mutagenic against *S. typhimurium* TA98, and two induce 'petite' mutants in *S. cerevisiae*.

Mutation in microbial systems

As summarized in Tables 1 and 2, compounds vary from highly active to inactive against the frameshift tester strain TA1537 (which detects frameshifts in a CCC sequence). None of the new antitumour compounds is active either against strain TA98, which generally detects frameshift events associated with DNA adduct formation [26], or against TA100, which detects base-pair substitutions [23]. Only some compounds are active 'petite' mutagens and these tend to be inactive as frameshift mutagens (Tables 1 and 2). Although amsacrine itself is not a 'petite' mutagen, there are three compounds (4, 12 and 13) which are moderately effective in the assay (Table 2).

Mutagenesis and micronucleus induction in V79 Chinese hamster cells

Data for cytotoxicity, clastogenicity and mutagenesis in the V79 6-thioguanine resistance resistance assay are summarized in Tables 1 and 2. The cytotoxic potential of the drugs varied widely, with D_{37} values ranging from 0.12 to 39.3 μM. None of the drugs induced statistically significant resistance at the ouabain locus in V79 cells despite analysis over a full dose curve, but all showed significant mutation at the 6-thioguanine resistance. Maximal values for the mutagenicity of all drugs were 10–20-fold that of the negative control at the drug concentration inducing 37% survival of cells. All drugs caused an increase in micronuclei, again to levels approx. 10–20-fold those of the negative controls, although necessary doses for this activity varied widely between the drugs. There was a clear correlation between mutagenicity and cytotoxicity, as shown in Fig. 2.

DISCUSSION

As has been previously discussed [3] it is possible to develop 9-anilinoacridine derivatives with high antitumour activity but low mutagenicity in common microbial systems. Of the newer amsacrine analogues with activity against experimental solid tumours, the two derivatives (3 and 4) show minimal mutagenicity in all the microbial assay systems (Table 1). The remainder are inactive as frameshift mutagens on strains TA98 and TA100, but have low activity in strain TA1537. Only some compounds (8 and 9) act as efficient 'petite' mutagens in yeast.

Mammalian mutagenicity appears not to be related to any of the parameters for microbial mutagenicity, nor to DNA binding constants. In general, those compounds binding weakly to DNA (association constants less than 10^6) have low microbial mutagenicity, while the remainder may have either frameshift mutagenicity in bacteria or 'petite' mutagenicity in yeast, but not both (Tables 1 and 2). Three compounds (12–14) are active against TA98 and may therefore act by forming DNA adducts

Table 1. Biological data for amsacrine and selected newer analogues

No.	Structure*	Substituents	NSC No.	DNA binding† $K \times 10^{-6}$	Lewis lung‡ OD (mg/kg)	Lewis lung‡ ILS (%)	Bacteria§ mutagen TA1537	Yeast‖ D_{37}	Yeast‖ Mutagen	V79 Cell¶ D_{37}	V79 Cell¶ Clastogen	V79 Cell¶ Mutagen
1	a	3′-OCH	156303	0.37	13.3	42 (0)	1.5	620	0	0.24	26	115
2	a	3′-OCH_3, 4-CH_3, 5′-$CONHCH_3$	343499	2.5	30	167 (2)	1.6	224	0	0.12	50	310
3	a	3′-$N(CH_3)_2$, 3-CH_3	600007	0.43	100	154 (2)	0.1	2040	0	0.31	12.8	56
4	a	3′-$N(CH_3)_2$, 4-OCH_3	600006	0.38	100	— (6)	0	1990	0	0.72	8.0	49
5	a	3′-$NHCH_3$, 4-CH_3	357499	4.0	20	139 (2)	2.3	1120	3	0.37	12.6	78
6	a	3′-$NHCH_3$, 3-CH_3, 4-CH_3	375158	9.1	20	155 (0)	4.2	900	1	0.08	28	240
7	b	4-CH_3	351914	5.6	13.3	41 (0)	8.5	10900	1	0.20	13	72
8	c	3′-$NHCH_3$	357501	2.2	30	112 (1)	0	1630	17	0.35	5.4	61
9	c	3′-$NHCH_3$, 3-Cl, 5-CH_3	375155	33	45	143 (4)	0.2	2960	69	0.51	10.6	41

*Structure as in Fig. 1 with indicated substituents.

†Association constant for poly[dA–dT] determined at 0.01 ionic strength by ethidium displacement.

‡Activity against i.v. inoculated Lewis lung carcinoma cells; OD = optimal dose; ILS = increase in lifespan over untreated mice; numbers in brackets indicate average numbers of long-term survivors in groups of six mice.

§Revertants/nmole of added drug; 0 = non-significant value. Values for TA98 and TA100 were non-significant in all cases.

‖D_{37} = concentration (μM) which reduces *S. cerevisiae* survival to 37%; mutagenicity = maximal 'petite' induction (%).

¶D_{37} = concentration (μM) which reduces V79 cell survival to 37%; clastogenicity expressed as gradient of regression line relating percentage of cells with micronuclei to drug concentration (μM); mutagenicity expressed as gradient of regression line relating frequency of thioguanine-resistant mutants to drug concentration (μM).

Table 2. Biological data for reference analogues of amsacrine

No.	Structure*	Substituents	DNA binding† $K \times 10^{-6}$	Bacterial mutagenicity TA98	TA100	TA1537	Yeast§ D_{37}	Mutagen	V79 Cell‖ D_{37}	Clastogen	Mutagen
10	a	—	1.5	0	0	12.5	330	0	0.32	23.8	102
11	a	3-NH_2, 6-NH_2	9.0	0	0	0.02	64	99	39.3	0.10	0.69
12	a	3′-OCH, 3-NO_2	0.45	5.5	0	0	620	0	0.55	7.4	38
13	a	3′-OCH_3, 5-$CONH_2$	0.30	0.3	0	0.1	510	43	0.43	5.5	32
14	a	3′-OCH_3, 3-NO_2, 5-$CONH_2$	0.16	6.1	0	0	1440	2	2.5	2.2	9.6
15	a	3′-OCH_3, 3-$NHCOOCH_3$	2.3	0	0	0	2840	1	0.60	5.1	27

*Structure as in Fig. 1 with indicated substituents.

†Association constant for poly[dA–dT] determined at 0.01 ionic strength by ethidium displacement.

‡Revertants/nmole of added drug; 0 = non-significant value.

§D_{37} = concentration (μM) which reduces *S. cerevisiae* survival to 37%; mutagenicity = maximal 'petite' induction (%).

‖D_{37} = concentration (μM) which reduces V79 cell survival to 37%; clastogenicity expressed as gradient of regression line relating percentage of cells with micronuclei to drug concentration (μM); mutagenicity expressed as gradient of regression line relating frequency of thioguanine-resistant mutants to drug concentration (μM).

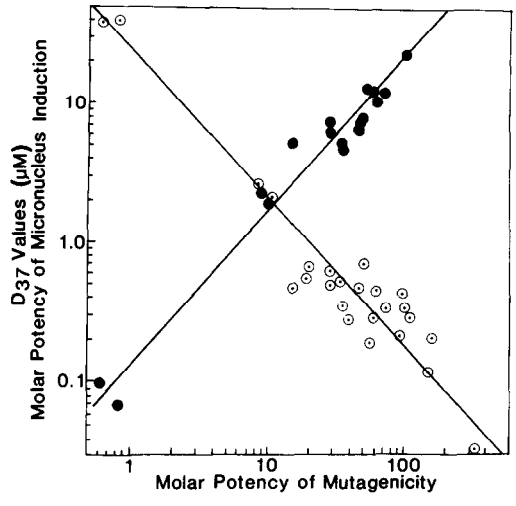

Fig. 2. Relationship between the molar potency of mammalian cell mutagenicity and either cytotoxicity (D_{37} values; open circles) or clastogenicity (molar potency of micronucleus induction) for compounds in Tables 1 and 2. Data differ from those in the tables in that individual values of mutagenicity are plotted, whereas they are averaged in Tables 1 and 2.

which then induce a frameshift mutation [26]. This might imply increased mutagenesis at the 6-thioguanine locus, which can be affected by point mutations as well as by chromosomal aberrations [27]. However, no increase was seen. Two of the compounds contain nitro groups, and it is possible that they are acted upon by nitroreductases present in bacterial but not in mammalian cells [28]. The reason for the activity of the third of these compounds, which has a carboxamide group, is not known.

The lack of correlation between 'yeast' induction and mammalian mutagenicity data suggests that fundamentally different mechanisms are operating. 'Petite' mutagenicity may result from mitochondrial concentration of drug [24], and it is interesting that compound 11, which has the highest 'petite' mutagenicity, is a much less potent mammalian mutagen than the corresponding compound (10) without the amino groups (Table 2) even though the two compounds have similar growth inhibitory activity towards V79 cells in continuous drug exposure growth inhibition assays [29]. It is possible that the growth inhibitory properties of this compound are derived mainly from mitochondrial effects whereas cytotoxicity arises from nuclear (topoisomerase II-mediated) damage.

The striking correlation between mutagenicity, micronucleus formation and cytotoxicity in mammalian cells (Fig. 2) suggests that all three processes are promoted by the same mechanism. The most likely mechanism involves the enzyme DNA topoisomerase II, which appears to mediate the cytotoxicity of amsacrine and many of its analogues [30]. It is interesting that corresponding data for Adriamycin and actinomyin D [10] places them on the regression line obtained for the amsacrine analogues, whereas data for 9-aminoacridine, the acridine alkylating agent ICR 191 and for ethyl methane sulphonate [10], which have different modes of action, do not fall on the regression line. Topoisomerase II has also been proposed as a target for amsacrine-induced cytotoxicity, clastogenicity and mutagenicity at the thymidine kinase locus of mouse lymphoma cells by DeMarini *et al.* [31]. If all of these processes are caused by the same mechanism, it may not be possible to produce anticancer drugs in the amsacrine series which are non-mutagenic to mammalian cells.

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