greater than that of plasma [6]. Moreover Table 1 suggests that all the drugs dissolve in the fat components of milk. The milk whey, which contains the proteins of the milk, does not bind the drugs. The increase in milk localization, when the ionized fraction of the drug is smaller at higher pH, strengthens the hypothesis that the drug undergoes liposolubilization in triglyceride micelles of milk.

Notwithstanding these considerations of lipid solubility, the less lipophilic drugs are more extensively concentrated in milk (Table 1). This suggests that plasma protein binding controls and impairs the drug transfer from plasma to milk. Accordingly, sotalol and acebutolol, which are the less hydrophobic beta-adrenoceptor antagonists, are weakly bound to plasma proteins and have the highest milk-to-plasma concentration ratios. In contrast propranolol, which is a lipophilic drug but strongly bound to plasma proteins, has a milk to plasma concentration ratio less than 1.

In addition the binding parameters in milk correlate strongly with those in plasma (Fig. 2). Consequently, drugs which have high *NK* values for milk are strongly bound in plasma and curiously not concentrated in milk. Thus, these findings suggest that β blockers strongly bound in plasma should be used preferentially in lactating women.

In summary, the binding of beta-adrenoceptor antagonists in milk has been studied by equilibrium dialysis. The results of our investigation indicate that the major determinant of the transfer of this class of drugs into breastmilk is plasma binding. Thus simple *in vitro* studies including measurement of the serum binding, may aid in the selection of the treatment of hypertension for lactating women in order to limit adverse effects on the suckling infants. Further investigations involving other classes of drugs are necessary to draw general guidelines for most drugs.

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Verapamil modulates mutagenicity of antitumour acridines in bacteria and yeast

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A variety of antibiotics and synthetic DNA binding antitumour drugs are excluded from mammalian cells by an energy-dependent outward efflux mechanism. Resistance [1] may be acquired to drugs such as Adriamycin, amsacrine and vincristine by a mechanism, possibly resulting from gene amplification [2], whereby the outward efflux of drug is increased, leading to a reduction of its intracellular concentration [3, 4]. This mechanism, known as multiple drug resistance, can be at least partially inhibited by the calcium channel antagonist verapamil [5]. It is unlikely that this effect is mediated directly by changes in cellular calcium ion concentrations, and current evidence points to a direct effect of verapamil on the drug efflux mechanism [6].

We have previously investigated the bacterial mutagenicity of antitumour 9-anilinoacridine derivatives in microbial cells [7–9]. We have found that strongly basic 9-anilinoacridines (i.e. those with a high pK_a) have a lower mutagenicity in bacteria than would be expected on the basis of their DNA binding properties. Furthermore these drugs have a high efficiency of production of respiratory deficient ("petite") mutants in yeast. The inverse correlation between bacterial mutagenicity and yeast mitochondrial mutagenicity suggests that a drug transport mechanism may be operating on one hand to exclude positively charged drugs from bacteria and on the other to concentrate them in yeast mitochondria [9]. We show here that verapamil partially overcomes resistance in cultured mammalian cells to some of these positively charged acridine antitumour drugs, increases their mutagenicity in bacteria and decreases their efficiency in forming "petite" colonies in yeast.

The compounds studied (I–III, see Fig. 1 for structures) are related to the drug amsacrine, which was first synthesized by Cain and Atwell [10] and has now found widespread use in the treatment of acute leukaemia [11]. The parent compound I [4′-(9-acridinylamino)methanesulphonanilide], which lacks the 3′-methoxy group of amsacrine, was chosen because of its high DNA binding activity and its higher mutagenicity in microbial systems [7–9]. Murine P388 leukaemia cells and Adriamycin-resistant P388/ADR leukaemia cells were grown in culture (ref. 12 and B. C. Baguley and W. R. Wilson, *Eur. J. Cancer clin. Oncol.*, in press) and the degree of cross-resistance of the drug-resistant line was determined for (I), its 3-amino derivative (II), and its 3,6-diamino derivative (III) in continuous drug exposure growth inhibition assays (Table 1).

Cross-resistance to the parent compound (I) was 5-fold, similar to that shown for amsacrine itself (6-fold). However, cross-resistance to the strongly charged amino derivatives was considerably higher (160-fold and 77-fold respectively for compounds II and III). This finding is consistent with other results in this laboratory that a number of derivatives of amsacrine with amino or substituted amino groups on the acridine ring are much less effective against the P388/ADR cell line (B. C. Baguley, unpublished data). Proflavine (IV), a positively charged aminoacridine which lacks

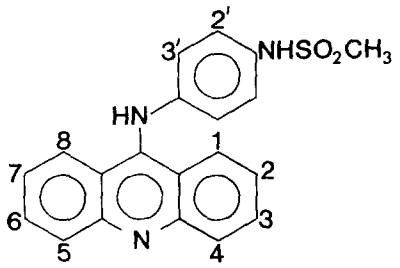

Fig. 1. Chemical structure of compound I. Compound II is the 3-monoamino derivative and compound III is the 3,6-diamino derivative. Proflavine (IV) is 3,6-diaminoacridine.

Table 1. Effects of acridine derivatives on growth of P388 and P388/ADR cells in culture

	IC_{50} value (nM)			
	P388		P388/ADR	
Drug	−Ver.*	+Ver.	−Ver	+Ver.
I	41 ± 4	33 ± 7	238 ± 63	196 ± 35
II	10 ± 4.1	10 ± 2.9	540 ± 93	82 ± 23
III	7.9 ± 0.9	7.0 ± 3.0	480 ± 48	89 ± 15
IV	650 ± 120	370 ± 80	1390 ± 470	950 ± 220

P388 and P388/ADR cell lines were obtained from Mason Research, USA, and passaged in mice before being adapted directly to tissue culture by methods which have been described previously [12]. Results are expressed as the mean and standard error of at least three experiments, each of which was carried out in duplicate.

* Verapamil (Ver) was added where indicated at a final concentration of 11 μM.

the anilino group (and the antitumour activity) of compounds I–III, was also tested. A small degree of cross-resistance was also observed (Table 1).

Verapamil at a concentration of 5 μg/ml (11 μM) had little effect on the *in vitro* growth inhibitory activity of compounds I–IV in the P388 cell line, and only a small effect on the activity of compounds I and IV in the P388/ADR cell line. However, it had a large effect on the sensitivity of the P388/ADR line to the amino derivatives II and III (Table 1). This is consistent with other findings [1, 4] that the P388/ADR cell line has a verapamil-sensitive drug efflux mechanism for (generally positively charged) intercalating antitumour drugs.

The bacterial mutagenicity of compounds I–IV was measured using a standard plate incorporation method [13]. As can be seen from the data in Table 2, the parent compound I was the most mutagenic in the frameshift tester strain TA1537. Proflavine was also highly mutagenic, whereas the amino derivatives II and III had low mutagenicity. The addition of verapamil had little effect on the maximal mutagenicity of drug I and of proflavine, although at low drug concentrations verapamil (50 μM) increased mutagenicity by up to 6-fold (results not shown). In contrast, for the amino derivatives II and III verapamil increased mutagenicity by 40- to 50-fold at low drug concentrations and also increased the maximal mutagenicity at the optimal drug dose (Table 2, Fig. 2). Verapamil also produced a slight increase in drug toxicity which may have masked an even greater mutagenic effect (Fig. 2). In the presence of verapamil, significantly lower concentrations of compounds II and III were required for a mutagenic effect. Verapamil by itself was neither mutagenic nor toxic to the bacteria under these conditions. Previous studies have shown that the toxicity of drugs related to I is correlated with drug lipophilicity [14] and the effect of verapamil on drug toxicity may well be related to membrane mediated toxicity.

The above results demonstrate that the pattern of response of bacteria to these acridine derivatives is similar to that of tumour cells with multiple drug resistance. Bacteria have probably developed drug efflux mechanisms for

Table 2. Effects of verapamil on mutagenicity of acridine derivatives in microbial systems

(a) *Salmonella typhimurium TA 1537*

	Mutagenicity at 0.1 μmole/plate Mutants/10^8 plated cells		Maximal mutagenicity Mutants/10^8 plated cells		Concentration per plate for 200 mutants/plate	
Drug	−Ver.*	+Ver.	−Ver.	+Ver.	−Ver.	+Ver.
I	270 ± 50	1190 ± 150	1480 ± 150	2100 ± 340	88 nmoles	49 nmoles
II	13 ± 4	640 ± 290	424 ± 82	1240 ± 170	610 nmoles	46 nmoles
III	9 ± 2	360 ± 34	19 ± 3	740 ± 30	—	60 nmoles
IV	28 ± 1	180 ± 100	1376 ± 85	983 ± 48	220 nmoles	104 nmoles

(b) *Saccharomyces cerevisiae D5*

	"Petite" mutagenicity at 10 μM % "petite" colonies		Maximal mutagenicity % "petite" colonies		Concentration for 50% "petite" colonies	
Drug	−Ver.*	+Ver.	−Ver.	+Ver.	−Ver.	+Ver.
I	0.5 ± 0.03	0.6 ± 0.2	0.8 ± 0.2	0.8 ± 0.5	—	—
II	17.5 ± 3.8	0.7 ± 0.1	71 ± 0.9	62 ± 7.7	23 μM	69 μM
III	61 ± 4	9.3 ± 4.4	99 ± 0.7	97 ± 0.6	7.5 μM	32 μM
IV	2.1 ± 1.1	1.1 ± 0.5	68 ± 1.7	68 ± 1.7	95 μM	190 μM

* Verapamil: added where indicated at same concentration as used in Figs. 2 and 3.

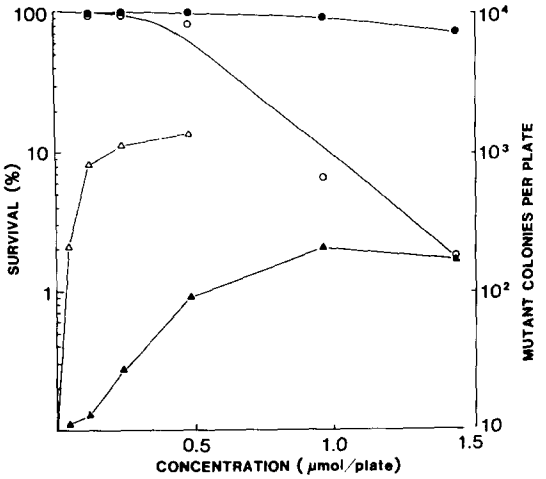

Fig. 2. Induction of bacterial frameshift mutagenicity by compound II in the presence or absence of verapamil. A range of concentrations of compound II, with and without 1.1 μmoles of verapamil, were made up in soft agar (2 ml) containing 5 mM histidine broth, and 100 μl (10^7 cells) of a growing culture of *Salmonella typhimurium* TA1537. This was spread over minimal agar plates [13], incubated for 3 days and colonies scored. Mutant colonies per plate were plotted against drug concentration with (△) or without verapamil (▲). A parallel experiment was performed to measure toxicity. Percentage survival was plotted against the dose of compound II with (○) or without (●) verapamil.

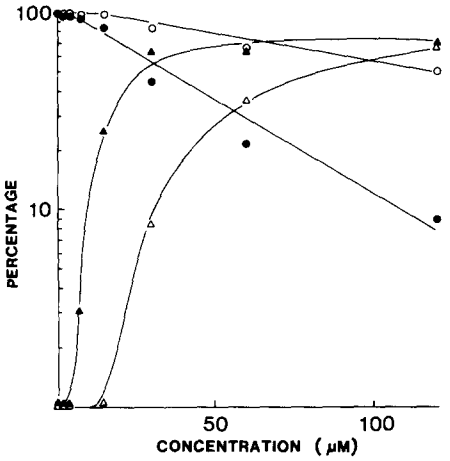

Fig. 3. Induction of yeast "petite" colony formation by compound II in the presence or absence of verapamil. Yeast cells were suspended at 4×10^4 cells/ml in YC medium, and after addition of different concentrations of compound II with or without verapamil (550 μM) were incubated at 28° for 24 hr. Cells were then washed, diluted appropriately and 100 μl samples plated on to YC plates which were incubated at 28° for 3 days. Colonies were counted, the percentage survival with (○) and without (●) verapamil calculated, and the percentage of "petite" colonies with (△) and without (▲) verapamil estimated using tetrazolium overlay [21].

excluding antibiotics produced by other microorganisms. They are known to contain a gene (*acr* A) which controls the mutagenic effects of acridine drugs though a membrane-mediated efflux mechanism [15]. This gene appears to be inoperative in bacterial cells infected with T-even bacteriophages [16], a finding consistent with the observations that anilinoacridine derivatives inhibit T-4 phage production [17], and that simple aminoacridines are highly mutagenic in T-even phage infected cells [18]. A reasonable explanation of the results in Table 2 and Fig. 2 is that verapamil inhibits drug efflux, thereby increasing mutagenicity.

Cultured yeast cells respond to a number of positively charged DNA binding drugs by the induction of respiratory deficient ("petite") mutants [19]. Because of the known propensity of mitochondria to accumulate basic dyes [20], one explanation of this behaviour is that the charged dyes are first accumulated by mitochondria and then exert their effects by binding to mitochondrial DNA. Solution assays of mitochondrial mutagenicity were carried out with *Saccharomyces cerevisiae* according to previously published methods [8] and the results are shown in Table 2 and in Fig. 3. Compounds II, III and IV were all active in this assay. The "petite" mutagenicity of the charged anilinoacridines II and III, and to a lesser extent proflavine was decreased by the addition of verapamil, although much higher concentrations are required than in bacteria or mammalian cells (Fig. 3). At the same time, toxicity of the drug, in terms of cell survival, was decreased by the presence of verapamil, as shown in the concentration curve for the effect of verapamil in Fig. 4. One possible explanation for this behaviour is that verapamil inhibits the uptake of drugs by yeast cells. Alternatively, verapamil may inhibit the mechanism whereby these basically charged drugs are concentrated in mitochondria. If the second explanation is correct, the results imply that drug toxicity may be mediated by a mitochondrial mechanism.

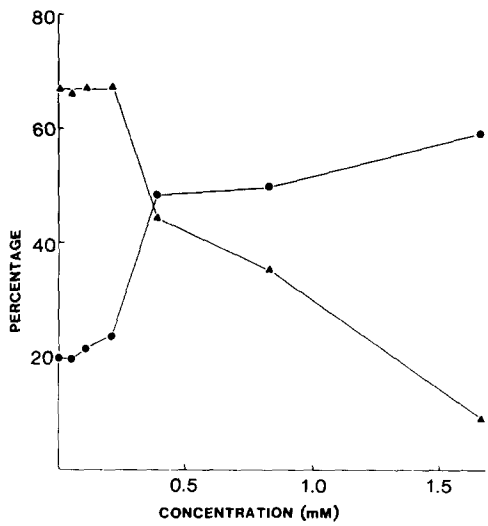

Fig. 4. Dose relationship for the effect of verapamil on yeast in the presence of compound II. Yeast cells were suspended at 4×10^4 cells/ml in YC medium containing compound II (48 μM) and a range of verapamil concentrations, and incubated at 28° for 24 hr. Cells were washed, diluted appropriately and 100 μl samples plated on to YC plates which were incubated at 28° for 3 days. Colonies were counted, percentage survival (●) calculated and the percentage of "petite" colonies (▲) esimated using tetrazolium overlay [21].

In conclusion, the calcium channel antagonist verapamil, which partially reverses multiple drug resistance properties exhibited by tumour cells, also reverses the resistance of Adriamycin-resistant P388 cells to positively charged 9-anilinoacridine derivatives related to amsacrine. In addition, it strongly increases the frameshift mutagenicity of these compounds in the Ames' test while decreasing the production of respiratory deficient "petite" mutants in yeast. These results suggest a similarity of microbial and mammalian cell mechanisms for the transport of positively charged DNA binding drugs. One implication of these results is that compounds which are effectively excluded by bacteria may be non-mutagenic (Ames' test) in the absence of verapamil but mutagenic in its presence. Whether this also applies to mammalian cells remains to be determined. Such a mechanism would have major implications in clinical medicine, where verapamil is in common use.

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Production of superoxide anion radicals during the oxidative metabolism of amino-chloramphenicol

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Therapeutic use of the antibiotic chloramphenicol (CAP*) is limited due to the potential occurrence of reversible hematopoietic depression and irreversible aplastic anemia [1]. The mechanism by which CAP causes aplastic anemia has yet to be elucidated, but reactive metabolites of the *p*-nitro moiety are thought to be involved [2, 3]. Among such metabolites, nitroso-chloramphenicol (NO-CAP) has received the most attention as a possible mediator of CAP-induced aplastic anemia [3–5]. Two major pathways have been proposed for the production of the NO-CAP reactive intermediate from the parent compound. In the first pathway, NO-CAP is thought to be produced during nitro-reduction of CAP to amino-CAP (NH_2-CAP); this could possibly occur in the intestine, liver, and/or bone marrow. Based on current experimental evidence, it is doubtful, however, whether any NO-CAP produced in the liver could accumulate and then be transported, in the unreacted form, to the bone marrow [6–8]. Possible CAP reduction in rat bone marrow has also been examined but was not detectable [4].

An alternative pathway for the *in vivo* production of NO-CAP involves the reduction of CAP to NH_2-CAP and subsequent oxidative metabolism of the compound in the liver and/or bone marrow. Amino-CAP is a known metabolite of CAP and has been isolated from many species including man [9–11]. In man, reduction mainly occurs in the liver, whereas the intestine is the major site for the rat and guinea pig [9–11]. Ascherl *et al.* recently reported that NH_2-CAP can be oxidatively metabolized by rat liver microsomes to NHOH-CAP [8]. Evidence for the toxicity of NHOH-CAP is scant, although under aerobic conditions some covalent binding to microsomal protein does occur [8]. An alternative mechanism to covalent binding by which the metabolism of aromatic amines may give rise to reactive intermediates is free radical formation [12]. In the present investigation we have examined the production of free radicals during the metabolism of NH_2-CAP by rat liver microsomes.

Materials and methods

Chloramphenicol, cytochrome *c* (horse heart; type VI), catalase (bovine liver; EC 1.11.1.6), xanthine oxidase (buttermilk; grade I; EC 1.2.3.2), peroxidase (horseradish; type II; EC 1.11.1.7), chloroperoxidase (EC 1.11.1.10), superoxide dismutase (bovine liver; EC 1.15.1.1), NADPH (type III), succinic anhydride, Trizma base, and xanthine (grade V) were obtained from the Sigma Chemical Co., St.

* Abbreviations: CAP, chloramphenicol; NO-CAP, nitroso-chloramphenicol; NHOH-CAP, hydroxylamino-chloramphenicol; NH_2-CAP, amino-chloramphenicol; PB, phenobarbital; BNF, beta-naphthoflavone; ANF, alpha-naphthoflavone; SFCC, succinoylated ferricytochrome *c*; and SOD, superoxide dismutase.