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Protection by Inhibitors of Multidrug Resistance against Mitochondrial Mutagenesis in *Saccharomyces cerevisiae*

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Abstract—*A number of inhibitors thought to act on the drug efflux mechanism of multidrug-resistant cells have been tested for their ability to inhibit the induction of respiration-deficient (petite) colonies of the yeast* Saccharomyces cerevisiae *by mitochondrial mutagens. The mutagens tested were* 3,6-*diamino*-9-{4-[(*methylsulphonyl*)*aminophenyl*]*amino*}*acridine (an antitumour compound related to both amsacrine and proflavine), ethidium bromide, quinolinium dibromide (NSC* 176319, *a non-intercalative DNA binding antileukaemia agent) and rhodamine* 123. *The inhibitors tested included verapamil, perhexiline, chlorpromazine, trifluoperazine, reserpine, chloroquine, quinacrine, tamoxifen, clomiphene, cyclosporin A, valinomycin, amphotericin B and Tween* 80. *Several of these agents protected against mitochondrial mutagenesis, the most active being verapamil, reserpine, chloroquine, cyclosporin A and Tween* 80. *The correspondence between activity against multidrug resistance and activity in the yeast system strongly implies some degree of similarity in mechanisms for drug efflux from multidrug-resistant cells and drug uptake into the mitochondria of yeast. Agents protecting against the uptake of drugs into mitochondria of mammalian cells may have use in minimizing the long-term toxicity of anticancer drugs mediated by mitochondrial drug retention.*

INTRODUCTION

TRANSMEMBRANE DRUG TRANSPORT is an important principle governing antitumour selectivity. It is particularly relevant to the action of antitumour agents such as the *Vinca* alkaloids, anthracyclines, actinomycins and epipodophyllotoxins, since an active cellular efflux mechanism may control intracellular drug concentrations [1]. The acquisition of transport-mediated multidrug resistance (MDR) in cancer cells is accompanied by an increase in the rate of drug efflux of all of the above drug categories and can lead to clinical drug resistance [2]. MDR can be inhibited by a wide variety of agents including verapamil, calmodulin inhibitors, cyclosporin, Tween 80, reserpine and many other agents [3–6]. These compounds partially or completely restore drug sensitivity in culture, and verapamil has been used clinically to combat MDR [7]. The pharmacological inhibition of MDR is an important concept in the optimization of cancer chemotherapy.

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One of the features of cells exhibiting MDR is the overexpression of one or more of a family of genes, termed *mdr* genes, which specify membrane proteins known as P-glycoproteins [8]. The DNA sequence of the mammalian *mdr*1 gene contains homologies with bacterial haemolysin transport gene [9], with a yeast *STE*6 protein transporter gene [10], and with a drug resistance gene from *Plasmodium falsiparum* [11], suggesting that it is one of a large family of transporter molecules. It is noteworthy that the bacterium *Salmonella typhimurium* TA1537 exhibits features of MDR in its response to certain acridine derivatives since frameshift mutagenicity is greatly increased in the presence of verapamil [12]. Furthermore, chloroquine-resistant variants of the malarial parasite *P. falsiparum* appear to have developed a type of MDR since sensitivity is enhanced in the presence of the calmodulin inhibitor desipramine [13].

Previous studies on mitochondrial mutagenesis of the yeast *Saccharomyces cerevisiae* by acridine derivatives have shown verapamil to have a paradoxical effect [12]. Rather than increasing the mutagenic effect of an antitumour acridine derivative, as might be expected if the yeast cell displayed the MDR phenotype, verapamil strongly suppresses muta-

genicity. Possible explanations are that verapamil inhibits the uptake of the acridine derivatives across the plasma membrane, that it stimulates drug metabolism or drug efflux, or that it inhibits uptake into mitochondria.

We have examined the hypothesis that yeast mitochondria have a verapamil-sensitive drug concentration mechanism with similarities to the drug concentration mechanism proposed for cytoplasmic vesicles of MDR cells. If this hypothesis is correct, then drugs known to reverse MDR in tumour cells might be expected to affect the action of antimitochondrial drugs. We have tested this hypothesis by examining the effects of a number of MDR-reversing agents on the induction of respiratory deficient ('petite') colonies of *S. cerevisiae* [12] by the following three mutagens. 3,6-Diamino-AMSA is a DNA intercalating drug related to amsacrine but with the acridine substitution pattern of proflavine [12]. Ethidium bromide is an example of a DNA intercalating compound which localizes strongly in the mitochondria of tumour cells [14] and has been used extensively in studies with yeast [15]. Quinolinium dibromide is a representative [16] of a class of bis-charged non-intercalative DNA-binding antitumour agents which also affect yeast [17]. A fluorescent member of this series has been shown to localize in tumour cell mitochondria [14]. Rhodamine 123 is a fluorescent laser dye which localizes in tumour cell mitochondria [18], affects yeast mitochondria [15], does not bind to DNA, and has selective cytotoxic effects against carcinoma cells [19].

MATERIALS AND METHODS

Compounds

3,6-Diamino-9-{4-[(methylsulphonyl)aminophenyl]amino}acridine (3,6-diamino-AMSA) was prepared in this laboratory as previously described [20]. Other materials were purchased from commercial sources as follows. Verapamil: Knoll AG; trifluoperazine dihydrochloride: Smith, Kline and French; clomiphene citrate and perhexiline maleate: Merrell; reserpine: Ciba-Geigy; chlorpromazine hydrochloride: Rhone-Poulenc; tamoxifen: Imperial Chemical Industries; cyclosporin A: Sandoz; chloroquine, valinomycin, nigericin, amphotericin, and quinacrine: Sigma.

Organisms

Saccharomyces cerevisiae diploid strain D5 [17] was kindly provided by Dr B.S. Cox (Botany School, University of Oxford). A single colony isolate was inoculated into liquid yeast complete medium (YC) [21] and grown to stationary phase for 24 h. Ten per cent dimethylsulphoxide was added, 1-ml aliquots were frozen to −70°C and stored at this temperature before use. For all experiments, a 1-ml sample was thawed, addded to fresh medium (10 ml) and grown for exactly 2 h before use.

Microtitre assay for 'petite' induction

This assay has previously been described [22]. Briefly, a logarithmic phase culture was diluted into fresh YC medium, 100-μl aliquots were inoculated into a 96-well microtitre tray (A/S Nunc, Denmark) and drugs were added at various dilutions to the wells. Drugs were dissolved in growth medium (Tween 80, verapamil, trifluoperazine, cyclosporin A, chlorpromazine, chloroquine), acetone (valinomycin), ethanol (perhexilene, clomiphene, quinacrine) or dimethylsulphoxide (reserpine, amphotericin B, tamoxifen) and dilutions made so that there was no more than 1% organic solvent per well. Trays were incubated for 20 h at 30°C, an appropriate dilution made from each well into saline, and 100 μl plated on to each of 10 YC plates. Appropriate solvent controls were also carried out. Cell numbers were calculated so that the dilutions at this point were at least 10^4-fold, thereby effectively washing drugs from cells by dilution. Plates were incubated at 30°C for 3 days before scoring for total colony counts, then overlaid with tetrazolium in order to score 'petite' colonies [22].

For most experiments, 'petite' colony induction by the drug alone was compared with that in the presence of 3,6-diamino-AMSA (4.7 μM). Variations in protocol are described within the text. All experiments were performed at least twice and each concentration was tested in triplicate.

RESULTS

Inhibition of mitochondrial mutagenesis by verapamil

In initial experiments concerned with inhibiting 'petite' mutagenesis by the calcium channel blocking agent verapamil, yeast cells were incubated with different concentrations of verapamil added 0, 0.5, 1 or 2 h prior to the addition of the 'petite' mutagen 3,6-diamino-AMSA. The concentration of mutagen was chosen either to induce 'petite' colonies (4.7 μM) or to kill cells (120 μM). Verapamil reduced both the 'petite' forming ability and toxicity of 3,6-diamino-AMSA and its efficiency was greatest with no preincubation step (Fig. 1). In all subsequent experiments test agents were added at the same time as the mutagen.

Inhibition of mitochondrial mutagenesis by other MDR-reversing agents

The efficiency of a range of test agents, selected to span a range of known activities in reversing MDR [3–6], was tested using the 'petite' mutagen 3,6-diamino-AMSA (4.7 μM). The known ionophore, valinomycin, was also tested because of its

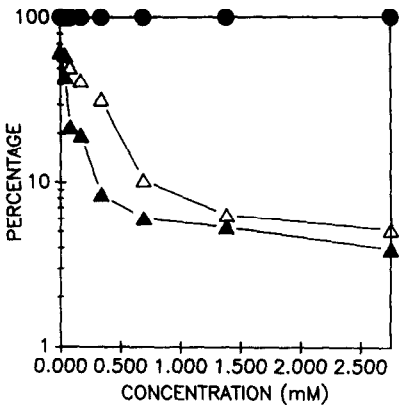

Fig. 1. Effects of adding verapamil at two different times on the reduction of 'petite' mutagenesis by 3,6-*diamino-AMSA* (4.7 μM). *Yeast cells were incubated with different concentrations of verapamil, added either before or at the same time as* 3,6-*diamino-AMSA. After addition of* 3,6-*diamino-AMSA cultures were grown for a further* 20 h *before plating for survival and 'petite' colony counting.* (○) *Percentage survival;* (△) *percentage 'petites' formed by* 3,6-*diamino-AMSA added* 2 h *after preincubation in the presence of various concentrations of verapamil;* (▲) *percentage petites formed by* 3,6-*diamino-AMSA added immediately after various concentrations of verapamil.*

effects on the mitochondrial uptake of rhodamine 123 [23]. Examples in Fig. 2 show valinomycin together with a highly active agent (cyclosporin A), and a weakly active agent (trifluoperazine). All curves were analysed to find the concentrations necessary to reduce survival and to reduce 'petite' mutagenesis to 50% of its original level. The maximum reduction of 'petite' formation in these experiments was also calculated. The data, summarized in Table 1, suggest that five compounds (reserpine, chloroquine, quinacrine, cyclosporin A and Tween 80) are all more active than verapamil in reversing 'petite' mutagenesis.

In order to rank the above five compounds, an alternative test procedure was utilized where each drug was tested at an optimal dose (as determined above) for its ability to reduce 'petite' formation by different concentrations of 3,6-diamino-AMSA (Fig. 3). Chloroquine and Tween 80 were found to reduce this activity over a wider dose range than any of the other drugs.

To determine whether 'petite' induction by other mutagens was susceptible to these agents, the muta-

Table 1. Ability of various compounds to protect against 'petite' mutagenesis in Saccharomyces cerevisiae *strain D_5 by* 3,6-*diamino-AMSA*

Compound	D_{37} (mM)	P_{50} (mM)	Max change (%)	P_{10} (mM)	D_{37}/P_{50}
Calcium channel blockers					
Verapamil	2.8	0.10	95	NA	28
Perhexiline	0.06	NA	NA	NA	—
Calmodulin inhibitors					
Chlorpromazine	0.030	0.008	66	NA	4.4
Trifluoperazine	0.016	NA	18	NA	—
Inhibitors of vesicular catecholamine uptake					
Reserpine	>4	0.026	100	0.059	>150
Antimalarials					
Chloroquine	>3	0.006	95	NA	>500
Quinacrine	2.3	0.019	100	NA	120
Antioestrogens					
Tamoxifen	0.12	NA	NA	NA	—
Clomiphene	0.86	NA	NA	NA	—
Immunosuppressive agents					
Cyclosporin A	>20	0.024	100	NA	>800
Ionophores					
Valinomycin	0.015	0.0016	92	0.002	9.4
Detergents					
Tween 80	>380	23	100	NA	>170
Antifungal agents					
Amphotericin B	0.0004	0.0010	82	NA	0.42

D_{37}: drug concentration required (μM) to reduce yeast colony numbers to 37% that of the untreated control. Note that with some drugs it was not possible to reach toxicity.

P_{50}: dose to reduce petite formation to 50% of the value for 3,6-diamino-AMSA alone.

Max change: $100 \times (a-b)/a$ where a = percentage petites induced by 3,6-diamino-AMSA alone and b = percentage petites induced by 3,6-diamino-AMSA plus test compound.

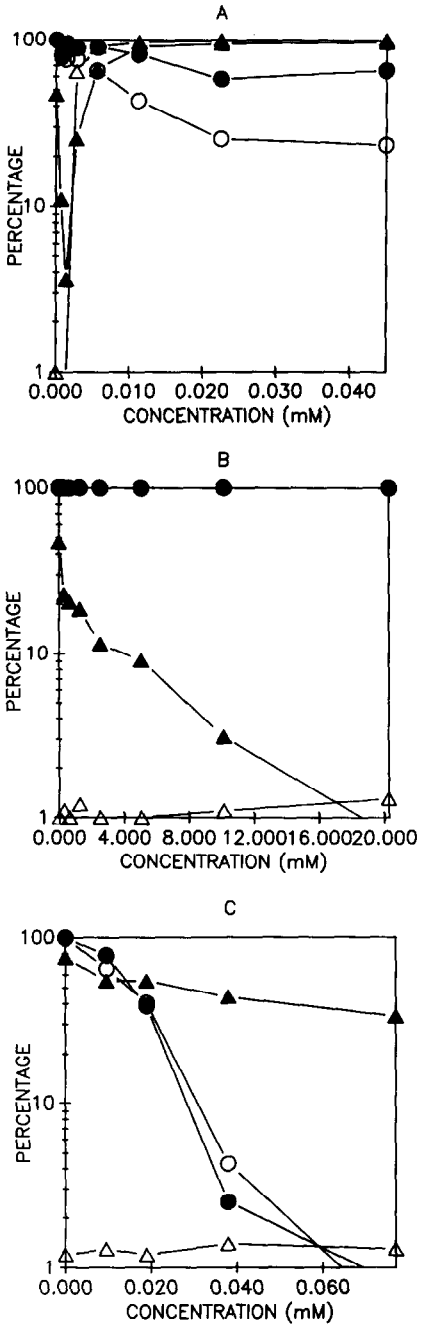

Fig. 2. Effect of valinomycin (A), cyclosporin (B) and trifluoperazine (C) on the survival and 'petite' mutagenesis of yeast in the presence (filled symbols) or absence (open symbols) of 3,6-*diamino-AMSA* (4.7 μM). *Both the mutagen and the protecting compound were added simultaneously and cultures grown for a further* 20 h *before plating for survival and 'petite' colony counting.* (○) *Percentage survival;* (△) *percentage 'petite' colonies.*

genicity of the DNA intercalator ethidium, the non-intercalative DNA binder quinolinium dibromide and the protein binder rhodamine 123 was tested in the presence of chloroquine, Tween 80 and verapamil. Optimal concentrations of the protecting compounds, determined from experiments above, were tested for their ability to reduce 'petite' mutagenesis (Table 2). Tween 80 was found to be the most effective agent, affecting the dose–response

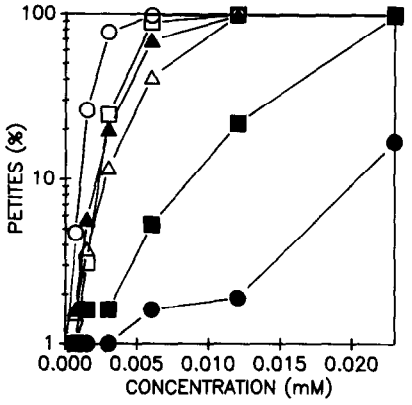

Fig. 3. Effects of various drugs on the ability of different concentrations of 3,6-*diamino-AMSA to induce 'petite' colonies. Both drugs were added simultaneously and cultures grown for* 20 h *before being plated for survival and petite colony counting.* (○) 3,6-*Diamino-AMSA alone;* (□) 3,6-*diamino-AMSA* + 16 μM *valinomycin;* (▲) 3,6-*diamino-AMSA* + 350 μM *reserpine;* (■) 2,6-*diamino-AMSA* + 400 μM *chloroquine;* (△) 3,6-*diamino-AMSA* + 400 μM *cyclosporin A;* (●) 3,6-*diamino-AMSA* + 47 mM (6.25%) *Tween* 80.

curve of all three agents. Chloroquine was ineffective against rhodamine 123 mutagenesis, while verapamil was ineffective against both ethidium bromide and rhodamine 123.

DISCUSSION

The surprising result in this study has been that many of the compounds known to reverse MDR in resistant cancer cells are able to protect yeast cells against the effects of the 'petite'-inducing mutagen 3,6-diamino-AMSA. This result extends to seven of the eight classes of agents studied, namely calcium channel blocking agents, calmodulin inhibitors, inhibitors of vesicular uptake of catecholamines, antimalarials, immunosuppressive agents, antifungals and non-ionic detergents (Table 1), and covers structures as diverse as cyclosporin A and reserpine. Verapamil is not the best of the agents studied, and ineffective against ethidium bromide and rhodamine 123. The activity of cyclosporin A and reserpine are notable.

Yeast cells [24], like mammalian cells [14, 18], are capable of concentrating cationic fluorescent dyes in their mitochondria. Although 3,6-diamino-9-anilinoacridine derivatives such as 3,6-diamino-AMSA are non-fluorescent, their concentration by mitochondria is implicated by the finding that, like ethidium [25], they strongly inhibit oxygen utilization by *S. cerevisiae* (Ferguson LR, Hill CL, unpublished). Although other mechanisms are possible, the simplest explanation for the results reported here is that compounds reversing MDR are capable of inhibiting the active concentration of 'petite'-inducing mutagens by yeast mitochondria.

The effect of valinomycin is interesting in that it can both suppress 'petite' mutagenesis by 3,6-diamino-AMSA at lower concentrations and induce

Table 2. Ability of Tween 80, chloroquine and verapamil to protect against 'petite' mutagenesis by ethidium bromide, quinolinium dibromide and rhodamine 123

'Petite' inducer	Concentration (μM)	Inhibitor	% 'Petites' (−)	(+)
Ethidium bromide	1.9	Tween 80	95	24
	1.9	Chloroquine	77	20
	1.9	Verapamil	98	96
Quinolinium dibromide	5.3	Tween 80	100	13
	5.3	Chloroquine	88	60
	5.3	Verapamil	88	45
Rhodamine 123	60	Tween 80	100	8
	60	Chloroquine	98	84
	60	Verapamil	99	99

it in the absence of a second agent at higher concentrations (Fig. 2 and Table 1). Valinomycin would be expected to abolish the mitochondrial membrane potential and thus disrupt mitochondrial energy production [23]. Complete disruption of mitochondrial function might be expected to lead directly to the formation of daughter cell buds lacking functional mitochondia, whereas partial inhibition may disrupt energy-dependent drug concentration by mitocondria. Reserpine may have analogous effects.

P-glycoproteins, which catalyse the export of cationic, hydrophobic drugs [1], appear to constitute a sub-population of a superfamily of translocator proteins which are required for the export of proteins from cells [10]. Since mitochondria contain specific translocator proteins for the import of mitochondrial proteins [26] it is reasonable to draw the analogy that one or more of these mitochondrial translocator proteins may catalyse the import of cationic, hydrophobic drugs. This mechanism is consistent with the finding that a variety of fluorescent, positively charged compounds known to accumulate in mitochondria of mammalian cells are also susceptible to MDR [14]. MDR appears to involve, as well as direct export of drug from the cell, the energy-dependent and verapamil-inhibitable accumulation of drug in cytoplasmic vesicles [27]. The active uptake of catecholamines into vesicles of cardiac atria may occur by an analogous mechanism since vesicular retention is inhibited by verapamil and reserpine [28]. The active uptake of drugs into mitochondria might be considered to be analogous to these processes.

In conclusion, it will be interesting to determine whether inhibitors of MDR protect against mitochondrial effects of drugs in tumour cells. The question of whether mitochondria have an inhibitable drug uptake mechanism is important because the antitumour effects [19, 29], as well as the long term toxic side-effects [29, 30], of some antitumour drugs may be due to mitochondrial localization. If mitochondrial drug accumulation can be controlled by a second agent, the selectivity of agents acting through mitochondria might be extended, and the delayed toxicity of other drugs minimized.

REFERENCES

1. Beck WT. The cell biology of multiple drug resistance. *Biochem Pharmacol* 1987, **36**, 2879–2888.
2. Beck WT, Cirtain MC, Lefko JL. Energy-dependent reduced drug binding as a mechanism of *Vinca* alkaloid resistance in human leukemic lymphoblasts. *Molec Pharmacol* 1983, **24**, 485–492.
3. Tsuruo R, Iida H, Tsukagushi S, Sakurai Y. Increased accumulation of vincristine and Adriamycin® in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. *Cancer Res* 1982, **42**, 4730–4733.
4. Ramu A, Glaubiger D, Fuks Z. Reversal of acquired resistance to doxorubicin in P388 murine leukemia cells by tamoxifen and other triparanol analogues. *Cancer Res* 1984, **44**, 4392–4395.
5. Tsuruo T, Iida H, Kitatani Y, Yokota K, Tsukagoshi S, Sakurai Y. Effect of quinidine and related compounds on cytotoxicity and cellular accumulation of vincristine and Adriamycin® in drug-resistant tumor cells. *Cancer Res* 1984, **44**, 4303–4307.
6. Slater LM, Sweet P, Stupecky M, Wetzel MW, Gupta S. Cyclosporin A corrects daunorubicin resistance in Ehrlich ascites carcinoma. *Br J Cancer* 1986, **54**, 235–238.
7. Presant CA, Kennedy PS, Wiseman C *et al.* Verapamil reversal of clinical doxorubicin resistance in human cancer: a Wiltshire Oncology Medical Group Pilot Phase I–II Study. *Am J Clin Oncol* 1986, **4**, 355–357.

8. Juliano RL, Ling V. Surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* 1976, **455**, 152–162.
9. Gerlach JH, Endicott JA, Juranka PF *et al.* Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. *Nature* 1986, **324**, 485–489.
10. McGrath JP, Varshavsky A. The yeast STE6 gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. *Nature* 1989, **340**, 400–404.
11. Foote SJ, Thompsen JK, Cowman AF, Kemp DJ. Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falsiparum. Cell* 1989, **57**, 921–930.
12. Baguley BC, Ferguson L. Verapamil modulates mutagenicity of antitumor acridines. *Biochem Pharmacol* 1986, **35**, 4581–4584.
13. Bitonti AJ, Sjoerdsma A, McCann PP *et al.* Reversal of chloroquine resistance in malaria parasite *Plasmodium falsiparum* by desipramine. *Science* 1988, **242**, 1301–1303.
14. Liley DTJ, Wiggins PM, Baguley BC. Localization of a nonintercalative DNA binding antitmour drug in mitochondria: relationship to multidrug resistance. *Eur J Cancer Clin Oncol* 1989, **25**, 1287–1293.
15. Wieker H-J, Kuschmit D, Hess B. Inhibition of yeast mitochondrial F_1-ATPase, F_0F_1-ATPase and submitochondrial particles by rhodamines and ethidium bromide. *Biochim Biophys Acta* 1987, **892**, 108–117.
16. Plowman J, Adamson RH. Initial studies on the disposition of quinolinium dibromide (NSC 176319) in mice and rats. *Pharmacology* 1978, **17**, 61–68.
17. Ferguson LR, Baguley BC. Induction of petite mutants in yeast by non-intercalative DNA-binding antitumour agents. *Eur J Cancer Clin Oncol* 1983, **19**, 1575–1583.
18. Johnson LV, Walsh ML, Chen LB. Localisation of mitochondria in living cells with rhodamine 123. *Proc Natl Acad Sci USA* 1980, **77**, 990–994.
19. Bernal SD, Lampidis TJ, McIsaac RM, Chen LB. Anticarcinoma activity *in vivo* of rhodamine 123, a mitochondrial-specific dye. *Science* 1983, **222**, 169–172.
20. Denny WA, Cain BF, Atwell GJ, Hansch C, Panthananickal A, Leo A. Potential antitumor agents. 36. Quantitative relationships between experimental antitumor activity, toxicity and structure for the general class of 9-anilinoacridine antitumor agents. *J Med Chem* 1982, **25**, 276–315.
21. Cox BS, Bevan EA. Aneuploidy in yeast. *New Phytol* 1962, **61**, 342–355.
22. Ferguson LR. Apparent changes in structure–activity relationships for antimitochondrial effects of 9-anilinoacridines according to *Saccharomyces cerevisiae* strain and methodology. *Mutation Res* 1984, **136**, 223–231.
23. Davis S, Weiss MJ, Wong JR, Lampidis TJ, Chen LB. Mitochondrial and plasma membrane potentials cause unusual accumulation and retention of rhodamine 123 by human breast adenocarcinoma-derived MCF-7 cells. *J Biol Chem* 1985, **260**, 13844–13850.
24. Williamson DH, Fennel DJ. The use of fluorescent DNA-binding agent for detecting and separating yeast mitochondrial DNA. *Methods Cell Biol* 1975, **12**, 335–351.
25. Peña A, Clemente SM, Borbolla N, Carrasco N, Uribe S. Multiple interactions of ethidium bromide with yeast cells. *Arch Biochem Biophys* 1980, **201**, 420–428.
26. Pfanner N, Hartl FU, Neupert W. Import of proteins into mitochondria: a multistep process. *Eur J Biochem* 1988, **175**, 205–212.
27. Horio M, Gottesman MM, Pastan I. ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proc Natl Acad Sci USA* 1988, **85**, 3580–3584.
28. Chaudry A, Vohra MM. Mechanism and metabolic disposition of verapamil-evoked overflow of radioactivity from isolated atria preloaded with [^{3}H]norepinephrine. *J Pharmacol Exp Therapeutics* 1985, **232**, 850–856.
29. Segal-Bendirdjian E, Coulaud D, Roques BP, Le Pecq J-B. Selective loss of mitochondrial DNA after treatment of cells with ditercalinium (NSC 335153), an antitumor bis-intercalating agent. *Cancer Res* 1988, **48**, 4982–4992.
30. Mimnaugh EG, Trush MA, Bhatnagar M, Gram TE. Enhancement of reactive oxygen-dependent mitochondrial membrane lipid peroxidation by the anticancer drug Adriamycin®. *Biochem Pharmacol* 1985, **34**, 847–856.