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The potential of *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide to circumvent three multidrug-resistance phenotypes in vitro

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Abstract The effectiveness of *N*-[2-(dimethylamino) ethyl]acridine-4-carboxamide (DACA) relative to that of amsacrine, idarubicin, daunorubicin and paclitaxel against three different forms of multidrug resistance (MDR) was determined using two sublines of the CCRF-CEM human leukaemia cell line, the P-glycoprotein-expressing CEM/VLB100 subline and the MRP-expressing CEM/E1000 subline, and two extended-MDR sublines of the HL60 human leukaemia cell line, HL60/E8 and HL60/V8. DACA was effective against P-glycoprotein-mediated MDR and MRP-mediated MDR, whereas the extended-MDR phenotype showed only low levels of resistance (< 2-fold) to DACA. In comparison, idarubicin was ineffective against the MRP and extended-MDR phenotypes. Repeated exposure of the K562 human leukaemia cell line to DACA (55, 546 or 1092 n*M* for 3 days over 10 weeks) did not result in the development of any significant drug resistance. We conclude that DACA has the potential to treat refractory leukaemia.

Key words Multidrug resistance · P-glycoprotein · MRP · DACA · Idarubicin · Paclitaxel

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Introduction

Multidrug resistance (MDR) is characterised clinically as a broad cross-resistance to chemotherapeutic drugs and is the main reason for the failure of chemotherapy. Whereas MDR is inherently expressed in some cancers, in others it develops in response to treatment. In vitro studies of drug-resistant cell sublines have been invaluable in identifying the molecular mechanisms involved in MDR. Overexpression of the *mdr*1 gene product, P-glycoprotein, produces an MDR phenotype characterised by decreased accumulation of drug and cross-resistance to lipophilic natural-product drugs [14]. P-glycoprotein is primarily located in the plasma membrane of drug-resistant cells, and it is believed that the decreased drug accumulation is the result of the adenosine triphosphate (ATP)-dependent drug-effluxing activity of P-glycoprotein.

Similarly, increased expression of the MDR-associated protein (MRP) causes resistance to a range of lipophilic natural-product drugs [6]. However, the cross-resistance profiles associated with MRP generally involve higher levels of resistance to etoposide and lower levels of resistance to vinblastine than those associated with P-glycoprotein [7, 8]. The molecular mechanism of MRP-mediated resistance has not yet been clearly defined. It is thought that MRP acts similarly to P-glycoprotein and causes drug efflux [7] since MRP has several features in common with P-glycoprotein, such as its sequence homology with *mdr*1, its location primarily in the plasma membrane and its association with decreased intracellular drug accumulation. Glutathione metabolism has been implicated in the MRP drug-resistance mechanism since buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, restores the cytotoxicity and the intracellular accumulation of drug in MRP-expressing cells [8]. It has been postulated that glutathionylated drug conjugates may be the substrate for MRP based on its ability

to transport the glutathione conjugate leukotriene C4 [16, 17]. As yet, glutathionylated MDR drugs have not been detected in association with MRP expression.

This increased understanding of MDR has stimulated the search both for compounds that circumvent MDR and for in vitro models of MDR in which these compounds can be tested. DACA, a lipophilic DNA-intercalating acridine derivative [1], is a dual-acting poison of the enzymes DNA topoisomerases I and II [11] that has strong activity against experimental solid tumours and has undergone clinical trial. DACA is capable of overcoming resistance in vitro to the P388 leukaemia line P/DACT, which exhibits characteristics of P-glycoprotein-mediated MDR [10, 22]. Moreover, DACA largely overcomes resistance in Jurkat leukaemia lines expressing low amounts of topoisomerase II, which are highly resistant to amsacrine, etoposide and doxorubicin [10]. However, the action of DACA against MDR cells needs to be characterised further as this will add to the understanding of its clinical potential.

Idarubicin has modes of action similar to those of DACA and is reported to be more effective than the parent anthracycline daunorubicin in the treatment of acute myelogenous leukaemia [3]. This has been attributed to its increased lipophilicity, which results in increased rates of intracellular accumulation and increased cytotoxicity [25]. Although idarubicin has been shown to be more effective than daunorubicin against P-glycoprotein-expressing cells [4], the potential of idarubicin against cells expressing MRP has not been determined.

In vitro models of MDR have been valuable in identifying strategies to circumvent these forms of drug resistance. We recently reported [8] the development of a series of drug-resistant sublines derived from the CCRF-CEM human leukaemia cell line by treatment with increasing concentrations of epirubicin. The level of drug resistance reflected the level of MRP-mRNA expression, and there was no detectable increase in expression of P-glycoprotein or *mdr1* mRNA in any of the sublines. One subline, designated CEM/E1000, was 94-fold resistant to epirubicin and showed a level of resistance to daunorubicin (51-fold [8]) similar to that of the well-characterised P-glycoprotein-expressing CEM/VLB100 subline (50-fold [8]) selected with vinblastine, also from the CCRF-CEM cell line [2]. Therefore, the CEM/E1000 and CEM/VLB100 sublines are the first examples of sublines derived from the same parental cell line with similar levels of drug resistance associated with two different drug-resistance mechanisms, MRP and P-glycoprotein, respectively.

The spectrum of drugs involved in the resistance caused by either P-glycoprotein or MRP is not as extensive as the broad cross-resistance experienced in the treatment of cancer, which, in addition to the lipophilic natural-product drugs, also includes resistance to alkylating agents, antimetabolites and the platinum-containing drugs. We recently described two drug-resistant sublines, HL60/E8 and HL60/V8, derived from the HL60 human leukaemia cell line, that not only were resistant to the lipophilic natural-product drugs but were also cross-resistant to chlorambucil, methotrexate, and cisplatin [24]. These sublines therefore provide a clinically relevant in vitro model displaying the same broad drug cross-resistance as that commonly encountered in the treatment of cancer.

The assessment of compounds to overcome MDR should also include determination of whether resistance develops to the new compound. We have shown that the K562 human leukaemia cell line readily adapts to repeated intermittent treatment with epirubicin and vinblastine, producing stable P-glycoprotein-expressing sublines that are 3- to 25-fold resistant to various MDR drugs [20]. We have also used the K562 cells to compare epirubicin, daunorubicin, doxorubicin and idarubicin for their ability to induce MDR. K562 cells treated repeatedly with a 20-ng/ml concentration of each of the anthracyclines for 3 days over a 2-month period showed that MDR was most likely to develop from epirubicin and daunorubicin treatment, which produced sublines that were 2.5- to 6.7-fold resistant to anthracyclines [15]. The K562 cell line is therefore well suited to assess the development of MDR.

Clinical MDR is probably caused by the co-expression of several different molecular mechanisms of drug resistance. Hence, we used a panel of sublines expressing three different MDR phenotypes, the P-glycoprotein-expressing CEM/VLB100 subline, the MRP-expressing CEM/E1000 subline and the extended-MDR HL60/E8 and HL60/V8 sublines, to determine the potential of DACA to treat MDR cancers relative to that of amsacrine, idarubicin, daunorubicin and paclitaxel. We also report the effect of repeated DACA treatment on the development of drug resistance in the K562 cell line.

Materials and methods

DACA and amsacrine were obtained from the Auckland Cancer Research Laboratory (Auckland, New Zealand), daunorubicin and idarubicin were purchased from Pharmacia (Sydney, Australia) and paclitaxel, BSO and verapamil were purchased from Sigma Chemical Company (St. Louis, Mo.). All other chemicals were of analytical reagent grade.

The CCRF-CEM cell line [12], the CEM/VLB100 subline [2], the CEM/E1000 subline [8], the HL60 cell line [13], the HL60/E8 and HL60/V8 sublines [24] and the K562 cell line [18] were maintained as suspension cultures at 37 °C in an atmosphere containing 5% CO_2 in RPMI-1640 medium supplemented with 20 m*M* HEPES, 10 m*M* sodium hydrogen carbonate and 10% fetal calf serum (Trace, Sydney, Australia). Exponentially growing cells were used for all experiments and all cells were free of mycoplasma.

A stock solution of paclitaxel was prepared in dimethylsulfoxide (1 mg/ml), and DACA and amsacrine were dissolved in 30%

ethanol/water to make stock solutions of 4 mg/ml. Idarubicin and daunorubicin were dissolved in water.

Sensitivity to drug was determined by exposure of cell cultures to drug for 4 days, after which their viability was measured using the MTT 3,-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide assay [19]. The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration causing a 50% reduction in cell viability. Relative resistance was calculated by dividing the IC_{50} obtained for the resistant subline by the IC_{50} obtained for the parental cell line. The presence of dimethylsulfoxide and ethanol had no effect on cell viability. Reversal of drug resistance was determined by incubation of cells in the presence or absence of either 10 μM verapamil, 10 μM BSO or 820 nM DACA in the cytotoxicity assay. The sensitisation ratio was calculated by dividing the drug IC_{50} obtained in th!e absence of sensitising agent by the IC_{50} obtained in the presence of sensitising agent. All cytotoxicity determinations were performed in triplicate and experiments were repeated at least three times. Significance testing was performed using the one-tailed *t*-test for unequal variance or the one-tailed paired *t*-test.

Selection for DACA resistance in the K562 cells was performed by treatment of cells for 3 days with 55, 546 or 1092 nM DACA, after which cells were transferred to drug-free fresh media. Treatment was repeated when the cells had recovered, and cells received up to ten cycles of treatment over a 3-month period.

The expression of MRP mRNA was determined by Northern blot analysis [23] using samples of 20 µg total RNA extracted by a guanidine thiocyanate method [5] and the *pmrp*10.1 probe, which was kindly provided by Dr. S. Cole [6].

Results

The order of increasing cytotoxicity (IC_{50}) of the five drugs for the CEM cells was $2{,}900 \pm 470$ nM DACA ($n = 4$), 360 ± 57 nM amsacrine ($n = 6$), 49 ± 15 nM daunorubicin ($n = 8$), 22 ± 8.7 nM idarubicin ($n = 6$) and 5.4 ± 0.5 nM paclitaxel ($n = 3$). The order was similar for the HL60 cells, with IC_{50} values being $1{,}400 \pm 300$ nM DACA ($n = 3$), 95 ± 40 nM amsacrine ($n = 3$), 27 ± 0.06 nM daunorubicin ($n = 3$), 12 ± 1.6 nM paclitaxel ($n = 4$) and 11 ± 4.1 nM idarubicin ($n = 3$).

Table 1 shows that DACA was the most effective drug against all three MDR phenotypes and that only the HL60/V8 subline showed a low (1.6-fold) but statistically significant level of resistance to DACA. In comparison, all cell sublines were resistant to amsacrine, idarubicin, daunorubicin and paclitaxel except for the HL60/V8 subline, which was not significantly resistant to paclitaxel. The CEM/E1000 subline was 29-fold resistant to idarubicin as compared with the 6.7-fold resistance of the CEM/VLB100 subline. Paclitaxel resistance was greatest (640-fold) in the CEM/VLB100 subline and lowest in the CEM/E1000 (1.7-fold) and HL60/V8 (1.6-fold) sublines. Based on the relative resistance, the order of effectiveness of the drugs for the P-glycoprotein-expressing CEM/VLB100 subline was DACA > idarubicin > amsacrine > daunorubicin > paclitaxel, whereas for the MRP-expressing CEM/E1000 subline it was DACA > paclitaxel > amsacrine > idarubicin > daunorubicin. For the HL60 sublines expressing the extended MDR phenotype the order of effectiveness of drugs for the HL60/E8 subline was more like the order found for the CEM/VLB100 subline, whereas the HL60/V8 subline was similar in this respect to the CEM/E1000 subline. However, these similarities in the order of effectiveness were not due to similarities in P-glycoprotein and MRP expression, since Fig. 1 shows that there was no significant difference in MRP mRNA in HL60/E8 and HL60/V8 sublines, both of which showed slightly higher levels than the HL60 parent cell line, and both sublines express similar levels of P-glycoprotein [24].

To determine whether sensitising agents would improve drug activity against P-glycoprotein- or MRP-mediated MDR, the effect of verapamil, an antagonist for both P-glycoprotein- and MRP-mediated resistance, and that of BSO, a sensitiser for MRP-mediated resistance, were determined. Table 2 shows that neither sensitiser increased DACA cytotoxicity in the CCRF-CEM line or in either of its drug-resistant sublines. The 29-fold idarubicin resistance associated with the CEM/E1000 subline was significantly reversed both by verapamil (sensitisation ratio 12.5) and by BSO (sensitisation ratio 6.1), whereas the 52-fold daunorubicin resistance of this subline was more effectively reversed by BSO (sensitisation ratio 10.9) than by verapamil (sensitisation ratio 4.6). The CCRF-CEM cells showed little response to these agents except for a 2.7-fold increase in idarubicin cytotoxicity in the presence of BSO.

Since DACA toxicity is not affected by P-glycoprotein or MRP expression, this compound may be capable of enhancing daunorubicin cytotoxicity in cells expressing P-glycoprotein or MRP by acting similarly

Table 1 Drug resistance of the sublines[a]

Cell subline	*x*-fold drug resistance relative to the parental cell line				
	DACA	Amsacrine	Daunorubicin	Idarubicin	Paclitaxel
CEM/VLB100	1.1 ± 0.2 (4)	8.6 ± 3.2 (5)[b]	59 ± 19 (10)[b]	6.7 ± 2.0 (6)[b]	640 ± 170 (4)[b]
CEM/E1000	1.1 ± 0.2 (4)	16 ± 8.8 (6)[b]	52 ± 14 (11)[b]	29 ± 10 (7)[b]	1.7 ± 0.3 (6)[b]
HL60/E8	2.0 ± 1.1 (3)	14 ± 6 (3)[b]	11 ± 4 (3)[b]	5.1 ± 2.5 (3)[b]	11 ± 4.8 (4)[b]
HL60/V8	1.6 ± 0.2 (3)[b]	4.2 ± 1.4 (3)[b]	8.2 ± 2.8 (3)[b]	14 ± 5.4 (3)[b]	1.6 ± 0.2 (4)

[a] The mean relative resistance ± SD (number of observations) is given
[b] Significantly different from the value recorded for the parental cell line

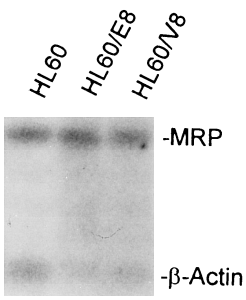

Fig. 1 MRP-mRNA expression as detected in the HL60 cell line and drug-resistant sublines. MRP mRNA was detected by Northern blotting and this was compared with β-actin mRNA to standardise for sample loading

to verapamil. To assess this possibility we included 820 n*M* DACA in a daunorubicin cytotoxicity assay for the CCRF-CEM cell line, the CEM/VLB100 and CEM/E1000 sublines. While 10 μ*M* verapamil sensitised both the CEM/VLB100 and the CEM/E1000 subline to daunorubicin (Fig 2B), DACA had no effect on daunorubicin cytotoxicity for any of the sublines tested (Fig. 2A).

The potential of DACA to induce drug resistance was determined by treating the K562 human leukaemia cell line with a range of DACA concentrations for 3 days each week for 10 weeks. As would be expected from the observed IC_{50} of 2,000 n*M* DACA for the K562 cells, treatment with concentrations of 1,092 n*M* DACA and below had no effect on the growth rate. Cells were monitored for resistance to DACA and for cross-resistance to daunorubicin during the 10-week period. Although no significant resistance developed, there was a trend towards low-level resistance, a typical example of which is shown in Fig. 3, whereby cells treated ten times with 540 n*M* DACA showed 1.6-fold resistance to DACA and 1.7-fold resistance to daunorubicin.

Discussion

DACA was the most effective drug against the MDR phenotypes represented in the panel of four MDR sublines since no significant difference in IC_{50} values was found between the CCRF-CEM, CEM/VLB100 and CEM/E1000 cells; the HL60/E8 subline was also not significantly resistant, whereas the HL60/V8 subline showed a low but significant 1.6-fold resistance to DACA. This low level of resistance to DACA may not be due to any specific resistance mechanism but could be related to the increased DNA content of the HL60/E8 and HL60/V8 sublines [24]. DACA is therefore capable of circumventing the action of P-glycoprotein and MRP, and it may also prove effective against the extended-MDR phenotype.

Our results (Table 1) confirm the previous reports of DACA being effective against P-glycoprotein-expressing cell lines [22]. Since the cytotoxicity of DACA is not affected by P-glycoprotein or MRP, this compound may also have the ability to block the drug efflux mediated by these glycoproteins similar to that shown by verapamil. Treatment of the CEM/VLB100 and the CEM/E1000 sublines with daunorubicin in the presence of a non-cytotoxic dose of 820 n*M* DACA did not increase daunorubicin cytotoxicity (Fig 2). This concentration of DACA was a 16-fold molar excess over the IC_{50} of daunorubicin, and it is therefore unlikely that DACA acts as a competitive inhibitor of P-glycoprotein activity or of MRP.

The IC_{50} values of 2,900 ± 470 n*M* DACA recorded for the CCRF-CEM cells and 1,400 ± 300 n*M* for the HL60 cells are higher than the respective values

Table 2 Effect of verapamil and BSO on drug sensitivity[a] (*NA* Not assayed)

Drug + sensitiser	Sensitisation ratio		
	CCRF-CEM	CEM/VBL100	CEM/E1000
DACA + verapamil	1.1	0.82	0.92
DACA + BSO	1.1	1.1	0.92
Amsacrine + verapamil	0.89	2.7[b]	1.4
Amsacrine + BSO	2.1	1.9[b]	1.8[b]
Idarubicin + verapamil	0.88	2.4[b]	12.5[b]
Idarubicin + BSO	2.7[b]	2.2[b]	6.1[b]
Daunorubicin + verapamil	1.3	13.1[b]	4.6[b]
Daunorubicin + BSO	1.3	2.2[b]	10.9[b]
Paclitaxel + verapamil	1.0	5.26[b]	1.6
Paclitaxel + BSO	NA	NA	NA

[a] Drug cytotoxicity (IC_{50}) was determined in the presence of either 10 μ*M* verapamil or 10 μ*M* BSO included in the culture media. The sensitisation ratio was calculated by dividing the IC_{50} obtained in the absence of sensitiser by the IC_{50} obtained in the presence of the sensitiser
[b] Significant sensitisation

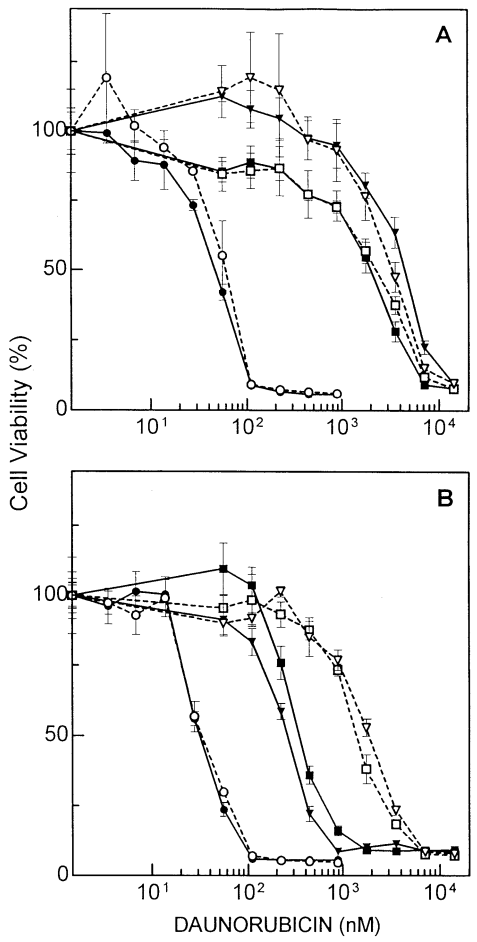

Fig. 2A, B Effect of DACA on daunorubicin cytotoxicity. The cytotoxicity of daunorubicin was determined in the CCRF-CEM (●, ○), CEM/VLB100 (▼, ▽) and CEM/E1000 (■, □) cell lines and sublines with no additives (*white symbols*) or in the presence of **A** 820 n*M* DACA (*black symbols*) or **B** 10 μ*M* verapamil (*black symbols*)

of 410 and 590 n*M* previously reported [10]. This difference may be due to the shorter drug exposure of 4 days used in the present study as compared with the 5 days used in the previous investigation. The IC_{50} values recorded for DACA for all cells were higher than the amsacrine, idarubicin and daunorubicin IC_{50} values. The low level cytotoxicity of DACA does not appear to jeopardise its clinical potential since plasma levels of 300–10,000 n*M* have been reported in rats [21].

The striking difference in paclitaxel cytotoxicity found between the CEM/VLB100 and CEM/E1000 sublines is noteworthy. Although relatively low levels of paclitaxel resistance in MRP-expressing cells have previously been reported [7, 27], these reports involved cells transfected with MRP that produced much lower levels of drug resistance than that expressed by the CEM/E1000 subline. However, both CEM sublines were 50-fold resistant to daunorubicin, yet the MRP-expressing subline showed less than 2-fold resistance to paclitaxel as compared with the 640-fold resistance observed for the P-glycoprotein-expressing subline. Being resistant to most natural-product drugs but being relatively sensitive to paclitaxel may be a useful means of distinguishing MRP-mediated drug cross-resistance patterns from those related to P-glycoprotein expression.

Another distinguishing feature of the MRP-mediated resistance was the high level of resistance to idarubicin (29-fold) as compared with that associated with P-glycoprotein expression (6.7-fold, Table 1). Treatment of leukaemia with combination regimens containing idarubicin is proving to be more effective than treatment with those containing daunorubicin [3]. The high level of resistance to idarubicin found in our MRP-expressing subline suggests that idarubicin may not have this advantage in patients with MRP-expressing leukaemias.

It is not clear as to how MRP causes idarubicin resistance. We show here that idarubicin resistance can be reversed by treatment with verapamil and with BSO (Table 2), which are known reversing agents of MRP-dependent resistance to MDR drugs [7, 8, 26]. However, we previously showed that there was no decreased cellular accumulation of idarubicin in the CEM/E1000 subline relative to the CCRF-CEM cells, whereas the CEM/VLB100 subline, which was less resistant to idarubicin, showed decreased cellular accumulation of idarubicin [8]. This suggests that MRP-mediated resistance may not always involve drug efflux.

We showed that DACA was effective against the three MDR phenotypes tested. However, there may be other MDR mechanisms not represented in our panel of sublines that cause resistance to DACA. Furthermore the clinical use of DACA may be limited by the development of resistance to DACA. However, our results suggest that drug resistance is less likely to develop in response to DACA treatment than to treatment with anthracyclines or vinblastine. We have previously shown that the K562 human leukaemia cell line easily adapts to treatment with epirubicin, daunorubicin, doxorubicin and vinblastine, and this leads to stable P-glycoprotein-expressing MDR sublines within 2 months [15, 20]. Treatment with DACA under conditions similar to those used in these previous studies did not produce any significant drug resistance (Fig. 3), even when DACA application was continued for a further four treatments.

The drug resistance encountered in the treatment of cancer is probably the result of a combination of several or many drug-resistance mechanisms. In our assessment of the potential of drugs to treat MDR cancer we used a panel of sublines of human leukaemia cells that represent three different MDR phenotypes: P-glycoprotein-mediated MDR, MRP-mediated MDR and extended MDR. Even though paclitaxel was effective against MRP-mediated drug resistance and idarubicin

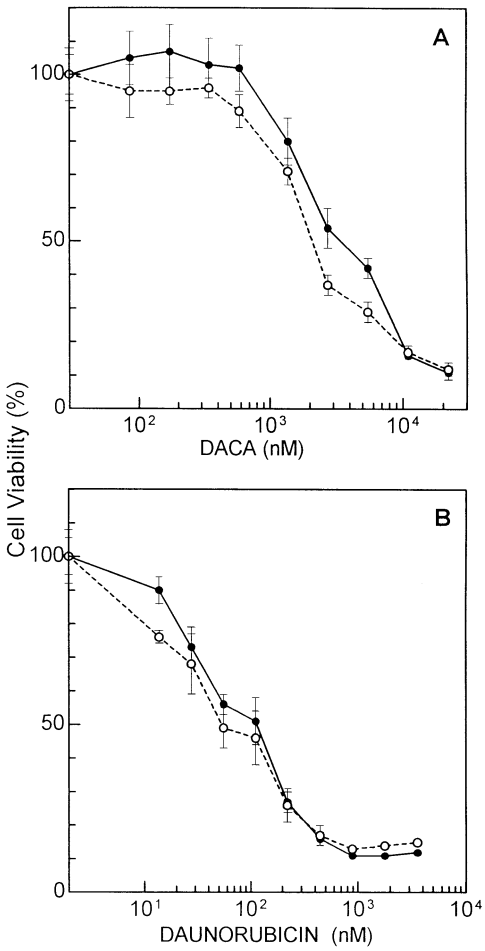

Fig. 3A, B Effect of selection with DACA on resistance to DACA in K562 cells. Cells were treated with 540 n*M* DACA ten times as described in Materials and methods and the cytotoxicity of DACA and daunorubicin was determined (*black symbols*) and compared with that found in untreated K562 cells (*white symbols*)

was relatively effective against P-glycoprotein-mediated resistance, DACA was the only drug effective against all the three phenotypes. This finding, along with the lack of development of drug resistance in the DACA-treated K562 cells, leads us to conclude that of the five drugs tested, DACA has the greatest potential to treat refractory leukaemia.

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