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IDENTIFICATION OF A MULTIDRUG RESISTANCE MODULATOR WITH CLINICAL POTENTIAL BY ANALYSIS OF SYNERGISTIC ACTIVITY *IN VITRO*, TOXICITY *IN VIVO* AND GROWTH DELAY IN A SOLID HUMAN TUMOUR XENOGRAFT

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Abstract—Circumvention of multidrug resistance *in vitro* by resistance modulators is well documented but their clinical use may be limited by effects on normal tissues. We have compared four resistance modifiers, both in terms of modulation of doxorubicin sensitivity *in vitro* and toxicity *in vivo*, in order to determine whether it is possible to select agents with clinical potential. Verapamil, D-verapamil and quinidine are all maximally active in the multidrug resistant cell line at about 7 μM and are not cytotoxic at this concentration. The tiapamil analogue Ro11-2933 is a highly potent resistance modulator such that at only 2 μM sensitization is greater than is seen with the other modulators at 7 μM. Since the ID_{50} concentration for Ro11-2933 is 17.7 μM (5–12-fold less than the other modifiers) we have used isobologram analysis to demonstrate that the interaction with doxorubucin is supra-additive and cannot be explained by additive toxicity. This method of analysis also revealed that when resistance modulation is related to the cytotoxicity of the modulator itself, all four modulators show comparable activity. On the other hand, measurement of the acute toxicity in mice of the modulators did reveal differences. The LD_{10} for verapamil (51 mg/kg) was about one third of that for quinidine (185 mg/kg) and this is consistent with the known maximum tolerated plasma levels in patients. Furthermore, whilst epirubicin alone was unable to reduce the growth rate of a multidrug resistant human tumour xenograft, the addition of quinidine, but not verapamil, at the maximum tolerated dose did do so. D-Verapamil was only about half as toxic as racemic verapamil and this too is consistent with clinical observations. The LD_{10} for Ro11-2933 (152 mg/kg) was comparable with that for quinidine. In the human tumour xenograft model maximal growth inhibition was observed with the combination of epirubicin and Ro11-2933 (45 mg/kg) and this degree of growth inhibition was comparable to that obtained with epirubicin alone in the drug sensitive xerografts. Ro11-2933 had no measurable effects on the plasma or tumour pharmacokinetics of epirubicin. These results suggest that it is possible to predict the clinical potential of a resistance modulator. Furthermore, Ro11-2933 is a promising agent for use in the clinic since maximal resistance modulation *in vivo* is observed at about one third of the LD_{10} dose.

Key words: Multidrug resistance, resistance modulators, synergism

Induction of drug resistance *in vitro* by chronic exposure of cells to a cytotoxic drug has allowed the identification of a number of potential resistance mechanisms. Of these the multidrug resistance phenotype and associated drug efflux pump, P-glycoprotein (P170), has received most attention [1]. It is now well established that P170 is present in a number of human tumour types [2] and in some cases expression has been shown to correlate with response to chemotherapy [3]. This evidence suggests that it is a mechanism relevant to clinical drug resistance that could be exploited in attempts to improve chemotherapy of selected tumour types.

A number of non-cytotoxic agents have been shown to reverse drug resistance *in vitro*, possibly by inhibition of the drug efflux mechanism [4]. We have shown that verapamil can sensitize a number of multidrug resistant cell lines to doxorubicin [5]. Although this effect was significant at a verapamil concentration of 1 μM, activity was maximal at about 6–7 μM. However, whereas this concentration is non-cytotoxic *in vitro*, comparable plasma levels are toxic *in vivo* due to the cardioactive properties of verapamil [6]. These properties are unrelated to its activity as a resistance modulator and are not observed in studies of multidrug resistant cells *in vitro* [7].

There have been a number of clinical trials of verapamil in addition to chemotherapy in cancer patients and in these the maximum plasma levels achieved without toxicity were around 0.5–2 μM [6, 8, 9]. Although plasma levels do not necessarily reflect tumour levels it is probable that the activity of verapamil in the clinic is limited by toxicity. Previously, we demonstrated that the D-isomer of verapamil alone was as effective as the racemic mixture in terms of resistance modulation *in vitro* [5]. Since the D-isomer was reported to be less cardiotoxic that the L-isomer [10–12] we suggested

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that use of D-verapamil in patients could increase the maximum tolerated dose and hence plasma levels. Clearly, a comparison of resistance modulators *in vitro* would not be able to discriminate between the clinical potential of racemic verapamil and D-verapamil.

Further clinical studies of resistance modulators depend on the identification of agents which are active at concentrations that are non-toxic in patients. Although there is a wide range of agents capable of reversing multidrug resistance *in vitro* [13] very few studies have attempted to show activity of these agents *in vivo*. This is due partly to the difficulty of establishing suitable animal models [14]. Transgenic mice, which express the human *mdr*-1 gene in their bone marrow, have been used to demonstrated that the gene confers resistance to the bone marrow which can be circumvented by resistance modulators [15]. Of those studies that have used tumour bearing mice the vast majority have used ascites models where the tumour is grown in the peritoneal cavity and drugs are administered intraperitoneally. These tumour models are inappropriate since they take no account of the problems of delivery of the agent via the vasculature to a tumour at a site distant from the site of drug administration.

Here we describe a comparison of four resistance modulators in terms of both their synergistic activity *in vitro* and their toxicity and growth delay *in vivo*. Two of these, verapamil and quinidine, are already in use in clinical trials. D-Verapamil has been used in humans but no information was available with regard to the maximum tolerated dose. The fourth compound, Ro11-2933, has never been used in the clinic but has previously been shown to be an effective modulator *in vitro* [16]. The results of a human tumour xenograft experiment illustrate the value of combining isobologram analysis of modulation *in vitro* with an estimate of toxicity *in vivo* as a means of predicting activity *in vivo*. Furthermore, Ro11-2933 has extremely promising clinical potential.

MATERIALS AND METHODS

Materials. Verapamil, quinidine sulphate and MTT† were obtained from the Sigma Chemical Co. (Poole, U.K.). Doxorubicin and epirubicin were obtained from Farmatalia (St Albans, U.K.). The tiapamil analogue Ro11-2933 was a gift from Hoffman-LaRoche AG (Basel, Switzerland) and D-verapamil was a gift from Knoll AG (Ludwigshafen, Germany).

Cell lines. The human ovarian carcinoma cell line A2780 and a drug resistant subline, 2780AD, were obtained from Dr R. F. Ozols (Fox Chase Cancer Centre, Pennsylvania, U.S.A.). They were maintained in Roswell Park Memorial Institute 1640 medium (Northumbria Biologicals, Cramlington, U.K.) containing glutamine (2 mM), foetal calf serum (10%) and insulin (0.25 mL). The resistant line was grown in the presence of doxorubicin (2 μM) and is known to express P-glycoprotein [17] It was grown in the absence of drug for 5 days before experiments.

Cytotoxicity assay. Drug sensitivity was determined by a tetrazolium based chemosensitivity assay as described previously [18]. Briefly, cells were plated out at a density of 8×10^2 (A2780) or 2×10^3 (2780AD) per well in 96-well flat bottomed plates (Linbro from ICN Biomedicals Ltd, Rickmansworth, U.K.) and allowed to attach and grow for 2–3 days. They were exposed to drug for 24 hr and then fed with fresh medium daily for 3 days. On the fourth day, cells were fed with medium containing HEPES buffer (10 mM) and MTT (50 μL, 5 mg/mL) was added to each well. Plates were incubated in the dark at 37° for 4 hr, medium and MTT removed and the MTT-formazan crystals dissolved in dimethyl sulphoxide (200 μL/well). Glycine buffer (25 μL/well, 0.1 M, pH 10.5) was added and the absorbance measured at 570 nm in a multi-well plate reader (Model 2550 EIA reader, Bio-Rad, Hemel Hempstead, U.K.).

For drug addition a serial dilution of eight concentrations of doxorubicin was prepared at twice the final concentration. These were then diluted 50:50 with either drug free medium or medium containing modifier, at twice the final concentration, before addition to the wells. Four wells were used for each drug concentration. Verapamil and quinidine were dissolved in phosphate-buffered saline. Ro11-2933 was solubilized in dimethyl sulphoxide before addition to culture medium. The final dimethyl sulphoxide concentration when added to cells was less than 1%.

Results are expressed in terms of the drug concentration required to kill 50% of the cells (ID_{50}) estimated as the absorbance value equal to 50% of that of the control untreated wells.

Isobologram analysis. Drug interactions were analysed by the isobologram method [19]. For construction of the isobolograms a relative drug concentration of 1 was defined as the ID_{50} concentration of the drug or modulator alone. Since doxorubicin produced near linear dose–response curves after transformation it was used as the first drug for estimation of the envelope of addivitity [20].

Drug accumulation. Cells were grown in 6-well plates (Nuclon from Life Technologies, Paisley, U.K.) for 3 days to give a final density of about 10^6 cells/well. The medium was removed, 4 mL of fresh medium added to each well and the plates allowed to equilibrate for 1 hr at 37° in an atmosphere of 2% CO_2 in air. [^{14}C]Doxorubicin and doxorubicin were added in 1 mL of medium to give a final concentration of 1 μM and 0.01 μCi/mL. At specified times, plates were placed on ice and the medium removed. Cells were washed twice with ice cold PBS (Dulbecco's A) and incubated for 5 min at 37° with 0.5 mL trypsin/EDTA (0.25%/1 mM). The contents of the well were transferred to scintillation vials, the wells washed with 0.5 mL of PBS and the wash added to the vials. Scintillation fluid (10 mL, Ecoscint from National Diagnostics, Somerville, NJ, U.S.A.) was added to each vial and the radioactivity determined in a Packard liquid scintillation counter

† Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline.

(Canberra Packard, Pangbourne, U.K.). Three wells were used for each time point and cell counts were determined from an additional 3 wells.

Human tumour xenograft. Cell lines A2780 and 2780AD were established as xenografts in athymic female nude (MF1 nu/nu) mice. Monolayer cultures were harvested with trypsin/EDTA and resuspended in PBS. About 10^7 cells were injected into the right flank of four mice. After 3 weeks the tumours were aseptically dissected and 1-mm^3 pieces transplanted subcutaneously into 80 mice. Animals were used for experiments after about 2 weeks when the tumours were about 0.7 cm^3. Tumour doubling time was about 2 days for both A2780 and 2780AD.

Animal toxicity studies. A dose ranging experiment was carried out in tumour bearing mice for each modulator (four mice/group). The dose intervals used were 10 mg/kg for verapamil (range 50–100 mg/kg), 25 mg/kg for quinidine (range 150–250 mg/kg) and 20 mg/kg for Ro11-2933 (range 140–250 mg/kg). LD_{50} and LD_{10} values were derived from probit analysis of the data.

Tumour growth delay in vivo. Tumour bearing mice were randomized into groups of six and tumour volume and body weight recorded. Mice were injected i.p. with the modulator at 10:00 and again at 14:00. Verapamil and quinidine were use at the maximum tolerated dose, 40 mg/kg and 150 mg/kg, respectively, and Ro11-2933 was used at various doses as specified. Epirubicin (10 mg/kg) was administered i.v. by a tail vein at 12:00. Tumour volumes were estimated by caliper measurements assuming spherical geometry (volume = $\pi/6 \times d^3$, where d is the mean diameter). Both tumour volumes and body weights were recorded daily at 10:00. All experiments were terminated once tumours reached a diameter of 3 cm.

At the end of the experiment a tumour was removed from an animal in the control group and a cell line re-established *in vitro*. The tumour was minced finely with scalpels and incubated overnight with collagenase (200 U/mL; Boehringer Mannheim, Lewes, U.K.) in complete culture medium. Cells were pelleted by centrifugation at 200 *g* for 5 min and resuspended in culture medium and plated out at a density of about $10^4/cm^2$ in 25-cm^2 culture flasks (Nunclon). Cells were used for detection of P-glycoprotein and cytotoxicity assays after 2 weeks.

Pharmacokinetic studies. Plasma and tumour levels of Ro11-2933 were determined at various times after a single i.p. injection (45 mg/kg) in tumour (2780AD) bearing mice. Blood was removed by cardiac puncture and centrifuged immediately. Plasma was stored at −70° until analysis and samples from four mice were pooled for each time point. Tumours were removed intact and frozen immediately in liquid nitrogen.

Ro11-2933 was determined by HPLC with a C18 μBondapack column and a mobile phase consisting of acetonitrile/H_2O/HCl/ammonia solution (45:54:0.5:0.5 v/v) buffered to pH 5 with formic acid. The flow rate was 2 mL/min and Ro11-2933 was detected at 229 nm with a retention time of 9 min.

Plasma was extracted with an equal volume of ice cold acetonitrile. Samples were mixed and centrifuged in an Eppendorf centrifuge for 5 min. Injection volume was 25–100 μL. Tumours were homogenized in mobile phase (1 mL per gram of tissue) and centrifuged at 400 *g* for 5 min. The supernatant was mixed with an equal volume of ice cold acetonitrile and centrifuged in an Eppendorf centrifuge for 5 min. Injection volume was 100 μL.

Plasma and tumour levels of epirubicin were determined at various times after a single i.v. injection (10 mg/kg) via a tail vein. One group of mice was treated with a single i.p. injection of Ro11-2933 2 hr before administration of epirubicin.

Plasma (0.2 mL) was extracted with 5 mL of chloroform:propanol (2:1). Tumours were homogenized in PBS (1 g to 9 mL). Silver nitrate (33% w/v) was added to 1 mL of the homogenate to precipitate the proteins and epirubicin was recovered from the mixture by vortexing with 5 mL of chloroform:propanol (2:1) for 20 min on a Buchler evaporator. Samples were centrifuged at 400 *g* for 15 min at 4° and the lower organic phase transferred to clean tubes and dried in a Buchler evaporator. The residue was resuspended in methanol (100 μL). Daunorubicin was added to all samples before extraction as an internal control.

Epirubicin was determined by HPLC with a C18 μ-Bondapak column and a mobile phase consisting of water:acetonitrile (70:30) adjusted to pH 4.0 with formic acid. The flow rate was 2.5 mL/min and epirubicin was detected by fluorescence at 560 nm with an excitation wavelength of 480 nm. Sample volume was 50 μL.

Detection of P-glycoprotein. P-glycoprotein expression was assessed by immunocytochemistry with the monoclonal antibody MRK16 as described previously [21].

Statistical analysis. Statistically significant differences were identified either by Student's *t*-test or by analysis of variance as appropriate.

Table 1. Cytotoxicity of doxorubicin and the resistance modulators in cell lines A2780 and 2780AD

Agent	ID_{50} (μM) A2780	2780AD
Doxorubicin	0.004 ± 0.011	5.0 ± 0.1
Verapamil	91.7 ± 2.4	213.0 ± 7.8
D-Verapamil	84.3 ± 2.9	87.3 ± 1.5
Quinidine	74.4 ± 5.5	152.0 ± 2.0
Ro11-2933	13.0 ± 0.7	17.7 ± 0.4

Cells were exposed to drug for 24 hr. Results are the means ± SEM of three experiments.

RESULTS

Cytotoxicity of the drugs

The cytotoxicity of doxorubicin and each of the resistance modulators is shown in Table 1. Cell line 2780AD is about 1000-fold resistant to doxorubicin but shows only a 2-fold cross resistance to verapamil and quinidine. Interestingly, no difference is observed in the sensitivity to D-verapamil. Both cell

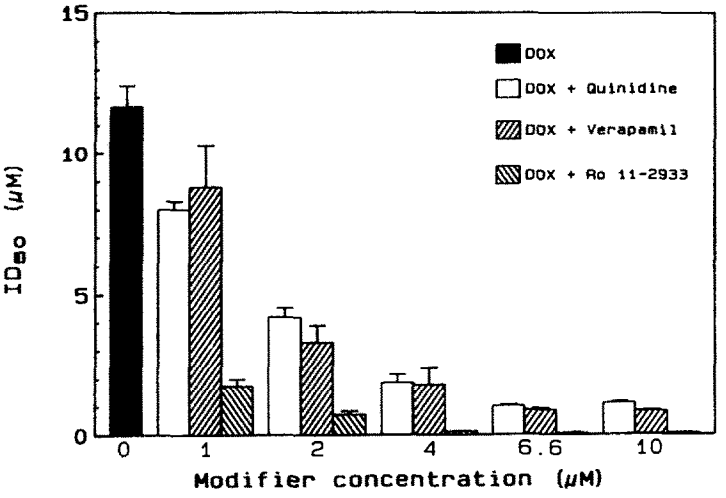

Fig. 1. Sensitivity to doxorubicin of cell line 2780AD in the absence (filled bar) and presence of various concentrations of quinidine (open bars), verapamil (hatched bars) or Ro11-2933 (hatched bars). The ID_{50} is the drug concentration required to kill 50% of the cells and results are the means ± SE of three observations.

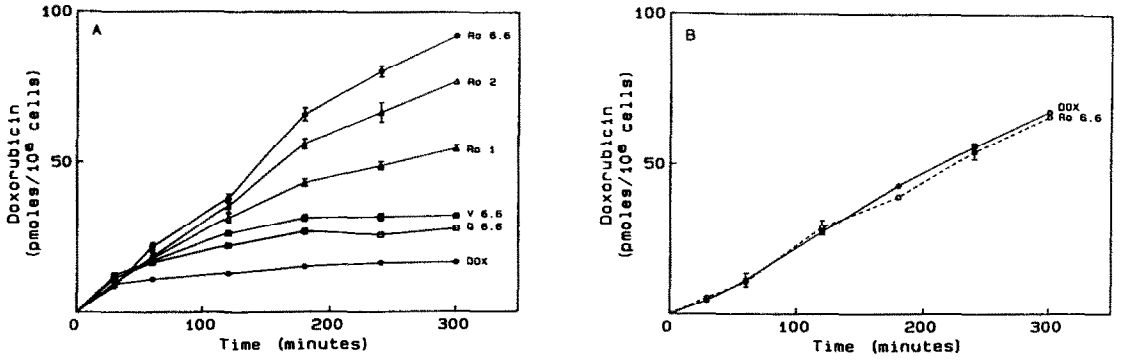

Fig. 2. Total cellular doxorubicin accumulation following incubation of cell lines (A) 2780AD and (B) A2780 in doxorubicin in the absence (●) and in the presence (○) of Ro11-2933 (6.6 μM). Also shown is the drug accumulation when 2780AD was incubated with drug in the presence of quinidine (□) and verapamil (■), both 6.6 μM, or Ro11-2933 (△, 1 μM; ▲, 2 μM). Points are the means ± SE of three observations.

lines are about 10-fold more sensitive to Ro11-2933 than to verapamil or quinidine.

Effects of resistance modulators on drug sensitivity

Figure 1 shows the sensitivity of 2780AD to doxorubicin alone and in the presence of various concentrations of verapamil, quinidine or Ro11-2933. All three modulators sensitize the cells to doxorubicin and this effect is concentration dependent. For verapamil and quinidine the effect is just significant at 1 μM ($P < 0.02$) and is significant at all higher concentrations ($P < 0.001$). Ro11-2933 has a highly significant effect on drug sensitivity at all concentrations used ($P < 0.001$). However, Ro11-2933 at only 1 μM increased the drug sensitivity to the same extent as achieved by either verapamil or quinidine at 6.6 μM. Furthermore, Ro11-2933 at 2 μM sensitized the cells to a much greater extent than could be achieved with verapamil or quinidine at concentrations as high as 10 μM.

Drug accumulation

Total cellular doxorubicin content of 2780AD incubation in doxorubicin (1 μM) for various times in the presence or absence of the resistance modulators is shown in Fig. 2a. For the parental cell line, A2780, drug accumulation increased linearly with time (Fig. 2b). However, for the resistant cell line accumulation reached a plateau by about 3 hr. After 5 hr A2780 had accumulated about four times as much drug as the resistant cell line.

The drug content of 2780AD was doubled in the

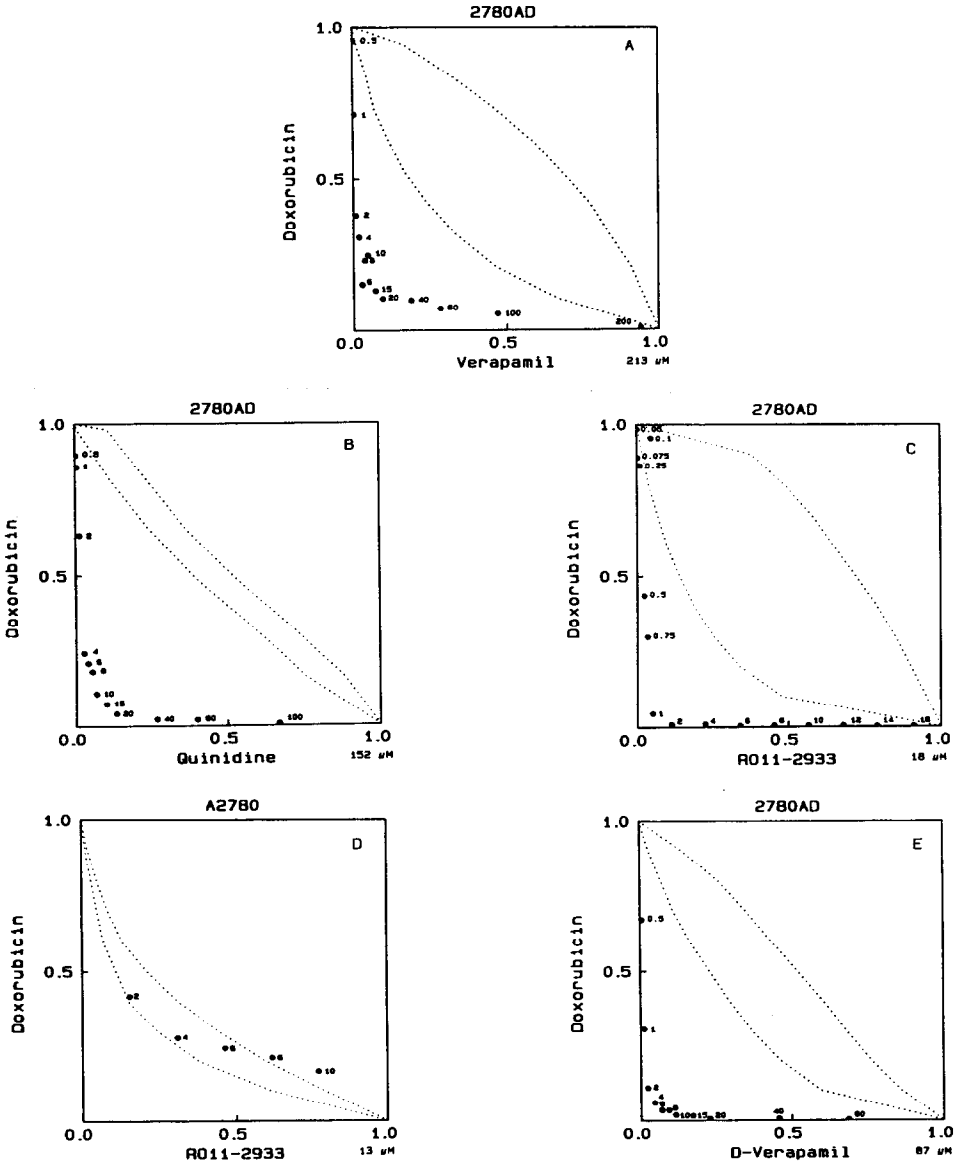

Fig. 3. Isobolograms for the interaction between doxorubicin and verapamil (A), quinidine (B), Ro11-2933 (C) and D-verapamil (E) in cell line 2780AD. Also shown is the isobologram for the interaction between doxorubicin and Ro11-2933 in cell line A2780 (D). The area between the dotted lines defines the envelope of additivity. The ID_{50} was used to define a relative drug concentration of 1 and for the modulators the actual concentration is shown on the x axis. The concentration (μM) of modulator used in each drug combination is shown adjacent to the point.

presence of verapamil (6.6 μM) and increased by about 70% in the presence of quinidine (6.6 μM). In contrast, drug accumulation did not show a plateau in the presence of Ro11-2933. After incubation for 5 hr with Ro11-2933 (1 μM) the cellular doxorubicin content was increased to 80% of that of A2780. It was increased to greater than that of A2780, 113% (2 μM) and 136% (6.6 μM), at higher concentrations.

Isobolograms

Isobolograms were constructed for doxorubicin in combination with each of the modulators (Fig. 3). For cell line 2780AD the isobolograms are similar and all points lie to the left of the envelope of additivity. This area is indicative of a supra-additive interaction, that is an interaction that is greater than would be expected based on the individual dose–response curves of the two drugs. In contrast, when cell line A2780 is exposed to doxorubicin and Ro11-2933 in combination the points lie either within the envelope of additivity or to the right of the envelope. Points to the right of the envelope indicate a sub-additive interaction.

Animal toxicity

The acute toxicity in tumour bearing mice of three

Table 2. Acute toxicity of the modulators in tumour bearing mice

Modifier	LD_{10} (mg/kg)	LD_{50} (mg/kg)
Verapamil	51 (32–81)	75 (62–92)
Quinidine	185 (164–207)	204 (191–219)
Ro11-2933	152 (129–180)	180 (160–203)

Drugs were administered i.p. and four mice were used at each dose. Confidence limits of 95% are shown in parentheses.

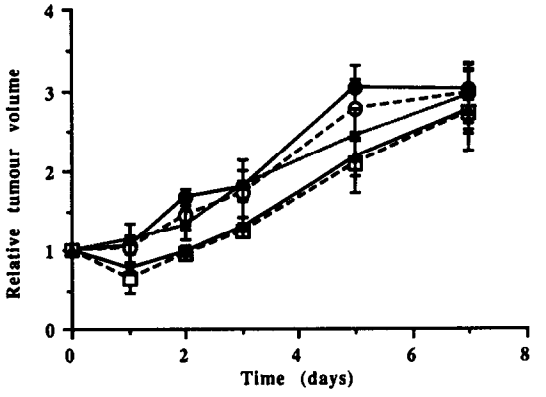

Fig. 4. Growth of the multidrug resistant human ovarian cell line 2780AD as a subcutaneous xenograft in nude mice. Groups of six mice received a single i.v. injection of epirubicin alone (--○--, 10 mg/kg) or were treated i.p. with verapamil (—×—, 40 mg/kg), quinidine (--□--, 150 mg/kg) or Ro11-2933 (—▲—, 30 mg/kg) 2 hr before and again 2 hr after injection of epirubicin. Mice were treated when tumours reached a mean diameter of about 0.5 cm and control groups were treated with PBS (—●—).

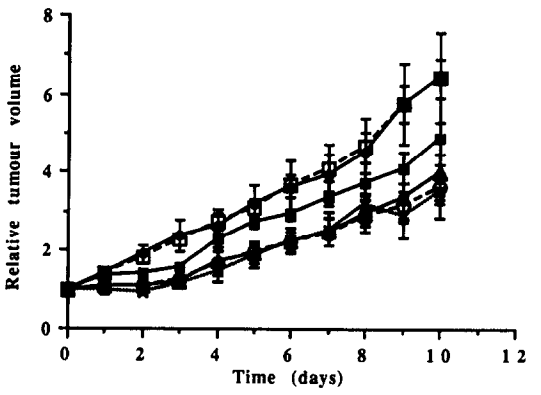

Fig. 5. Growth of the multidrug resistant human ovarian cell line 2780AD as a subcutaneous xenograft in nude mice. Groups of six mice received a single i.v. injection of epirubicin alone (—●—, 10 mg/kg) or were treated i.p. with Ro11-2933 at 30 (—■—), 45 (--○--), 60 (—▲—) or 75 (···×···) mg/kg 2 hr before and again 2 hr after injection of epirubicin. Mice were treated when tumours reached a mean diameter of about 0.5 cm and the control group (--□--) were treated with PBS.

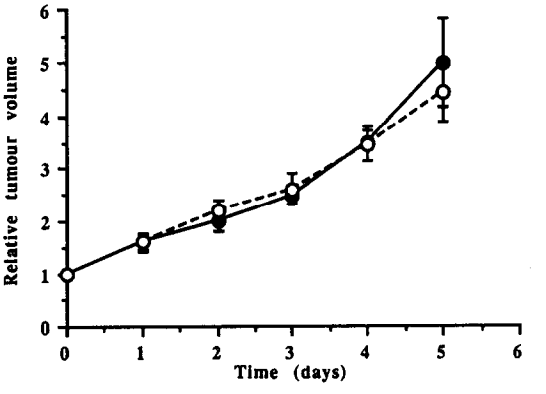

Fig. 6. Lack of effect of Ro11-2933 alone on the growth of the multidrug resistant xenograft. Mice received two i.p. injections, separated by 4 hr, of Ro11-2933 (--○--, 45 mg/kg). Control mice (—●—) were treated with PBS containing DMSO (10%).

of the modulators in shown in Table 2. For verapamil and quinidine deaths were observed within the first hour after administration of the drug. In contrast, no immediate toxicity was observed with Ro11-2933 and all deaths occurred between 3 and 24 hr after drug administration.

For D-verapamil, lethal seizures were observed in all mice at a dose of 200 mg/kg and in one of four mice at a dose of 150 mg/kg. At a dose of 120 mg/kg non-lethal seizures were observed in all mice. No such toxicity was observed at a dose of 100 mg/kg.

Tumour growth delay

The effect of epirubicin alone and in combination with resistance modulators on the growth rate of the multidrug resistant human tumour xenograft 2780AD is shown in Fig. 4. Epirubicin (10 mg/kg) alone had no effect on the growth rate of the xenograft. Furthermore, administration of verapamil at the maximum tolerated dose (40 mg/kg) 2 hr before and again 2 hr after treatment with epirubicin had no effect on the growth rate. In contrast, the combination of epirubicin with either quinidine at the maximum tolerated dose (150 mg/kg) or Ro11-2933 at about a fifth of the LD_{10} dose caused a significant ($P < 0.01$) reduction in the growth rate. Maximal growth delay was obtained with a combination of epirubicin and Ro11-2933 at a dose of 45 mg/kg ($P < 0.001$; Fig. 5). This concentration of Ro11-2933 alone had no effect on the growth rate of the xenograft (Fig. 6) nor did the higher concentrations. None of the treatments caused a significant loss of body weight.

Cells from a tumour taken from one of the control, untreated group were re-established in culture. Both the tumour and these cultured cells retained expression of P-glycoprotein (results not shown) but

Table 3. Effect of resistance modulators on the doxorubicin sensitivity of the multidrug resistant cell line established from a xenografted tumour of cell line 2780AD

Modulator	ID_{50} (nM)	Modulator factor
None	295 ± 2.9	—
Verapamil (6.6 μM)	27.0 ± 1.5	10.9
Quinidine (6.6 μM)	70.0 ± 3.5	4.2
Ro11-2933 (2 μM)	15.3 ± 0.9	19.3

Cells were exposed to doxorubicin for 24 hr either alone or in the presence of verapamil (6.6 μM), quinidine (6.6 μM) or Ro11-2933 (2 μM). Values are the mean ± SEM of three estimations and the modulation factor is the ID_{50} for doxorubicin alone divided by the ID_{50} for doxorubicin in the presence of the modulator.

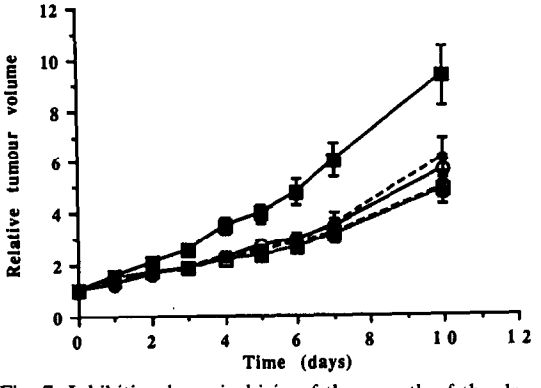

Fig. 7. Inhibition by epirubicin of the growth of the drug sensitive human ovarian cell line A2780 as a subcutaneous xenograft in nude mice. Groups of six mice received a single i.v. injection of epirubicin alone (—○—, 10 mg/kg) or were treated i.p. with verapamil (···□···, 40 mg/kg), quinidine (--▲--, 150 mg/kg) or Ro11-2933 (--●--, 45 mg/kg) 2 hr before and again 2 hr after injection of epirubicin. Mice were treated when tumours reached a mean diameter of about 0.5 cm and the control group (—■—) were treated with PBS.

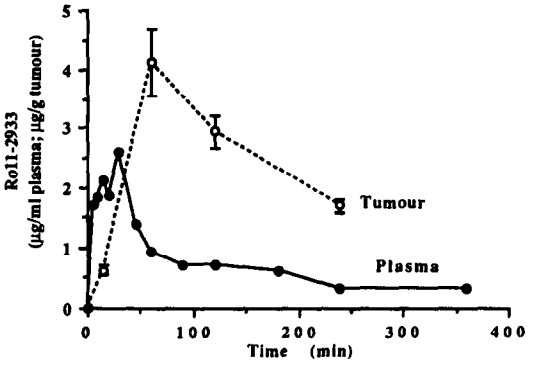

Fig. 8. Plasma (—●—) and tumour (···○···) levels of Ro11-2933 in mice bearing the 2780AD xenocraft. Mice were injected i.p. with Ro11-2933 (45 mg/kg) and samples were taken at various times. Values for plasma were obtained from pooled plasma from four mice and for the tumours, the values are the means ± SEM of tumours from four mice.

were about 17-fold more sensitive to doxorubicin than cell line 2780AD. However, the cells still showed enhanced sensitivity to doxorubicin when exposed in the presence of the resistance modulators (Table 3).

Epirubicin alone caused a significant ($P < 0.001$) reduction in the growth rate of the drug sensitive xenograft A2780 (Fig. 7). Addition of the modulators at the concentration which caused maximal growth delay in the drug resistant xenograft did not enhance the effects of epirubicin.

Pharmacokinetics of Ro11-2933 and epirubicin

Plasma and tumour levels of Ro11-2933 measured in samples taken at various times from mice treated i.p. with a single injection of Ro11-2933 at a dose of 45 mg/kg are shown in Fig. 8. In the growth delay experiments epirubicin was administered 2 hr after the modulator. At this time plasma levels of Ro11-2933 were 0.7 μg/mL (1.3 μM) and significant amounts were present in the tumours (2.9 μg/mg).

The effects of Ro11-2933 on the pharmacokinetics of epirubicin in mice bearing the multidrug resistant xenograft (2780AD) are shown in Fig. 9. There was no difference between either plasma (Fig. 9A) or tumour (Fig. 9B) levels of epirubicin in mice treated with epirubicin alone and those treated with epirubicin 2 hr after a single i.p. injection of Ro11-2933 (45 mg/kg).

DISCUSSION

These results demonstrate clearly that when compared *in vitro* at equimolar concentrations, Ro11-2933 is a more potent resistance modulator than verapamil or quinidine. However, when modulatory activity is related to the toxicity of the

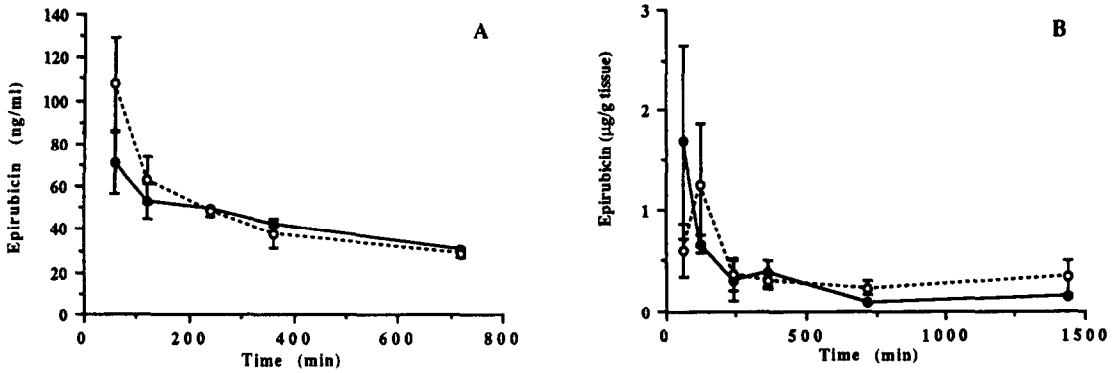

Fig. 9. Plasma (A) and tumour (B) levels of epirubicin in mice bearing the 2780AD xenograft treated i.v. with epirubicin (10 mg/kg) alone (—●—) or with epirubicin given 2 hr after a single i.p. injection of Ro11-2933 (···○···, 45 mg/kg). Values are the means ± SEM of samples from four mice.

agent *per se* by isobologram analysis then all four modulators appear similarly potent. The multidrug resistant human tumour xenograft 2780AD is resistant to epirubicin and when administered at the maximum tolerated dose verapamil does not sensitize the tumour to epirubicin. In contrast, quinidine and Ro11-2933 both sensitize the tumour to epirubicin and for Ro11-2933 this is achieved at only one third of the maximum tolerated dose.

Both verapamil and quinidine (6.6 μM) sensitize the multidrug resistant cell line 2780AD to doxorubicin by about 10-fold and no further increase in sensitization is observed at 10 μM (Fig. 1). A similar degree of sensitization was reported previously for D-verapamil [5]. Since the ID_{50} concentrations of verapamil, D-verapamil and quinidine alone in this cell line are 213, 87 and 152 μM, respectively, it is probable that these compounds are not cytotoxic in their own right at 6.6 μM and that the sensitization seen is due to a synergistic interaction with doxorubicin. Certainly there is no detectable growth inhibition when cells are exposed to verapamil, D-verapamil or quinidine at this concentration for 24 hr. Although these modulators have similar effects on drug sensitivity, accumulation of doxorubicin is doubled in the presence of verapamil (6.6 μM) or D-verapamil [5] but is increased by only 75% in the presence of quinidine (6.6 μM; Figs 2A). This disparity between effects on drug sensitivity and drug accumulation is often observed [22–24] and is further demonstrated by the activity of Ro11-2933. At a concentration of 1 μM, drug sensitivity is increased by about 5-fold and drug accumulation by about 3-fold; a much greater increase than is seen with verapamil or quinidine (6.6 μM). When cell line 2780AD was incubated with Ro11-2933 (6.6 μM) drug sensitivity increased by about 100-fold, drug accumulation was greater than observed for the parental cell line A2780, yet the sensitivity of 2780AD was still 10-fold less than that of A2780. This increase in drug accumulation to greater than that for the parental cell line does not appear to be due to a general cytotoxic action of Ro11-2933 since the same concentration had no effect on drug accumulation in cell line A2780 (Fig. 2B).

One problem encountered with the use of Ro11-2933 *in vitro* was that at a concentration of 6.6 μM a small growth inhibition was observed. This is confirmed by the observation that the ID_{50} of Ro11-2933 for A2780 and 2780AD was only 12 and 18 μM, respectively. Thus, part or all of the apparent modulatory activity might be explained simply by additive toxicity between Ro11-2933 and doxorubicin. A previous study reported that at a concentration of 2 μM, Ro11-2933 could sensitize cells to doxorubicin [25]. The authors reported that at this concentration exposure of cells to Ro11-2933 alone for 2 hr resulted in a 6% growth inhibition and for a 72 hr exposure a 26% growth inhibition was observed. They suggested that the longer exposure time was required for complete reversal of resistance in highly resistant cell lines. Clearly, this could have been explained by a combination of resistance modulation properties and of cytotoxicity of Ro11-2933. Therefore, we carried out a detailed isobologram analysis of the drug interactions (Fig. 3). This method of analysis allows the distinction between supra-additive and sub-additive drug interactions and clearly the interactions between the modulators and doxorubicin are supra-additive (i.e. synergistic). However, what is also apparent is that all four modulators are highly effective in terms of supra-additive activity *in vitro*.

Neither the simplistic comparison of equimolar concentrations of modulators nor the detailed isobologram analysis gives any indication of their clinical potential. It has already been demonstrated that the clinical potential of verapamil is probably limited by its cardioactive properties since the maximum tolerated dose in patients produces plasma levels of only 1–2 μM [6, 8, 9]. In contrast, at the maximum tolerated dose of quinidine (250 mg b.d.), plasma levels of 6 μM can be achieved in the clinic [26]. Clearly, the toxicity in patients is not reflected by the cytotoxicity of the compounds *in vitro* since quinidine is more cytotoxic than verapamil.

We have determined the acute toxicity of the modulators in tumour bearing mice. Although this is not a direct measure of the maximum tolerated dose even in mice it is possible that such a measurement can give an indication of the expected

maximum tolerated dose in humans. Verapamil is about 3-fold more toxic than quinidine and this agrees with the clinical observation that higher plasma levels of quinidine can be tolerated. Furthermore, although the acute toxicity of D-verapamil was about half that of racemic verapamil it was apparent that even in mice the maximum tolerated dose was limited by non-lethal toxicity. This result did not reflect the expected 10-fold decrease in cardiotoxicity reported for the D-isomer [27]. However, in a Phase 1 trial of D-verapamil in addition to doxorubicin in cancer patients the maximum tolerated dose of D-verapamil was only 600 mg/kg compared with 480 mg/kg for racemic verapamil. At this dose peak plasma levels of only 1.6 μM were achieved [28]. Hence, for the three modulators for which clinical information is available, the mouse toxicity results are a fair indication of the maximum tolerated dose in patients. It is, therefore, encouraging that the LD_{10} for Ro11-2933 in mice is 152 mg/kg. In a previous study plasma levels of Ro11-2933 of 2 μM were achieved in mice with a fifth (30 mg/kg) of this dose [29]. At this concentration Ro11-2933 was more active as a resistance modulator *in vitro* than verapamil or quinidine at 10 μM.

These results predicted that both quinidine and Ro11-2933 should sensitize multidrug resistant tumours to anthracyclines *in vivo* and our observations with the human tumour xenografts confirmed these predictions. For these studies epirubicin was used since it is less cardiotoxic than doxorubicin [30]. Xenografts of the multidrug resistant cell line 2780AD were resistant to epirubicin and verapamil was unable to sensitize the tumour even at the maximum tolerated dose (Fig. 4). In contrast, quinidine was able to sensitize the tumours to epirubicin. We have shown previously that at the dose of quinidine used (150 mg/kg) plasma levels of about 2 μg/mL (5 μM) and tumour levels of about 6 μg/g are achieved at the time of epirubicin administration [31]. The combination of Ro11-2933 (30 mg/kg) and epirubicin produced a growth inhibition comparable to that achieved with quinidine (Fig. 4) but maximal growth inhibition was achieved with Ro11-2933 at a dose of 45 mg/kg (Fig. 5). The mice tolerated Ro11-2933 at doses of 60 and 75 mg/kg but at these concentrations no further increase in growth inhibition was observed. Ro11-2933 alone was inactive (Fig. 6) and none of the modulators enhance the activity of epirubicin in the drug sensitive xenograft A2780 (Fig. 7). These observations suggest that the effects of the resistance modulators are related to the multidrug resistant phenotype of the xenograft and we have shown that a cell line derived from the xenograft retains this phenotype (Table 3). Furthermore, the maximum growth inhibition obtained in the multidrug resistant xenograft is comparable to that obtained by epirubicin alone in the drug sensitive xenograft (Fig. 5 cf Fig. 7) which suggests that the effectiveness of the combination of Ro11-2933 and epirubicin is limited by the activity of epirubicin. We have also demonstrated that the effect of Ro11-2933 could not be explained by an effect on the pharmacokinetics of the cytotoxic drug as has been suggested for verapamil [8].

Use of the isobologram analysis allowed the identification of Ro11-2933 as a potent resistance modulator *in vitro*. This method of analysis was particularly useful for this compound since Ro11-2933 is itself cytotoxic. Whilst such observations are important they do not give any indication of clinical value of the modulators. Our observations suggest that the inclusion of an estimate of the maximum tolerated dose in mice provides valuable information with regard to the clinical potential of the modulator. Furthermore, our results with the human tumour xenograft suggest that Ro11-2933 is a particularly promising agent for clinical studies of resistance modulation.

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REFERENCES

1. Endicott JA and Ling V, The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu Rev Biochem* **58**: 137–171, 1989.
2. Goldstein LJ, Galski H, Fojo A, Willingham M, Lai S-L, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM, Lieber M, Cossman J, Gottesman MM and Pastan I, Expression of a multidrug resistance gene in human cancers. *J Natl Cancer Inst* **81**: 116–124, 1989.
3. Chan HSL, Thorner PS, Haddad G and Ling V, Immunohistochemical detection of P-glycoprotein. Prognostic correlation in soft tissue sarcoma of childhood. *J Clin Oncol* **8**: 689–704, 1990.
4. Tsuruo T, Lidu H, Tsukagoshi S and Sakurai Y, Increased accumulation of vincristine and adriamycin in drug-resistant P388 tumour cells following incubation with calcium antagonists and calmodulin inhibitors. *Cancer Res* **42**: 4730–4733, 1982.
5. Plumb JA, Milroy R and Kaye SB, The activity of verapamil as a resistance modifier *in vitro* in drug resistant human tumour cell lines is not stereospecific. *Biochem Pharmacol* **39**: 787–792, 1990.
6. Ozols RF, Cunnion RE, Klecker RW, Hamilton TC, Ostchega Y, Parrillo JE and Young RC, Verapamil and adriamycin in the treatment of drug-resistant ovarian cancer patients. *J Clin Oncol* **5**: 641–647, 1987.
7. Ramu A, Spanier R, Rahamimoff H and Fuks Z, Restoration of doxorubicin responsiveness in doxorubicin-resistant murine leukaemia cells. *Br J Cancer* **50**: 501–507, 1984.
8. Kerr DJ, Graham, J, Cummings J, Morrison JG, Thompson GG, Brodie MJ and Kaye SB, The effect of verapamil on the pharmacokinetics of adriamycin. *Cancer Chemother Pharmacol* **18**: 239–242, 1986.
9. Dalton W, Grogan TM, Meltzer PS, Scheper RJ, Durie BGM, Taylor CW, Miller TP and Salmon SE, Drug-resistance in multiple myeloma and non-Hodgkins lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. *J Clin Oncol* **7**: 415–424, 1989.
10. Curtis MJ and Walker MJA, The mechanism of action of the optical enantiomers of verapamil against ischaemia-induced arrhythmias in the conscious rat. *Br J Pharmacol* **89**: 137–147, 1986.
11. Echizen H, Brecht T, Neidergesass S, Vogelgesang B and Eichelbaum M, The effect of dextro-, levo- and racemic verapamil on atrioventricular conduction in humans. *Am Heart J* **109**: 210–217, 1985.

12. Triggle DJ and Swamy VC, Calcium anagonists. Some chemical-pharmacological aspects. *Circ Res* **52** (Suppl 1): 17–28, 1983.
13. Ford JM and Hait WN, Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42**: 155–199, 1990.
14. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62**: 385–427, 1993.
15. Galski H, Sullivan M, Willingham MC, Chin K-V, Gottesman MM, Pastan I and Merlino GT, Expression of a human multidrug resistance cDNA in the bone marrow of transgenic mice: resistance to daunomycin-induced leukopenia. *Mol Cell Biol* **9**: 4357–4363, 1989.
16. Kessel D and Wilberding C, Promotion of daunorubicin uptake and toxicity by the calcium antagonist tiapamil and its analogs. *Cancer Treat Rep* **69**: 673–676, 1985.
17. Sugawara I, Kataoka I, Morishita Y, Hamada H, Tsuruo T, Itoyama S and Mori S, Tissue distribution of P-glycoprotein encoded by a multidrug resistance gene as revealed by a monoclonal antibody MRK16. *Cancer Res* **48**: 1926–1929, 1988.
18. Plumb JA, Milroy R and Kaye SB, Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Res* **49**: 4435–4440, 1989.
19. Steel GG and Peckham MJ, Exploitable mechanisms in combined radiotherapy-chemotherapy: The concept of additivity. *Int J Radiat Oncol Biol Phys* **5**: 85–91, 1979.
20. Steel GG, Terminology in the description of drug-radiation interactions. *Int J Radiat Oncol Biol Phys* **5**: 1145–1150, 1979.
21. Wishart GC, Plumb JA, Going JJ, McNicol AM, McArdle CS, Tsuruo T and Kaye SB, P-Glycoprotein expression in primary breast cancer detected by immunocytochemistry with two monoclonal antibodies. *Br J Cancer* **62**: 758–761, 1990.
22. Goldenberg GJ, Wang H and Blair GW, Resistance to adriamycin: relationship of cytotoxicity to drug uptake and DNA single- and double-strand breakage in cloned cell lines of adriamycin-sensitive and -resistant P388 leukaemia. *Cancer Res* **46**: 2978–2983, 1986.
23. Louie KG, Hamilton TC, Winker MA, Behrens BC, Tsuruo T, Klecker RW, McKoy WM, Grotzinger KR, Myers CE, Young RC and Ozols RF, Adriamycin accumulation and metabolism in adriamycin-sensitive and -resistant human ovarian cancer cell lines. *Biochem Pharmacol* **35**: 467–472, 1986.
24. Schuurhuis GJ, Broxterman HJ, Cervantes A, van Heijningen THM, de Lange JHM, Baak JPA, Pinedo HM and Lankelma J, Quantitative determination of factor contributions to doxorubicin resistance in multidrug-resistant cells. *J Natl Cancer Inst* **81**: 1887–1892, 1989.
25. Jamali MAA, Yin Mb, Mazzoni A, Bankusli I and Rustum YM, Relationship between cytotoxicity, drug accumulation, DNA damage and repair of human ovarian cells treated with doxorubicin: Modulation by the tiapamil analogue RO11-2933. *Cancer Chemother Pharmacol* **25**: 77–83, 1989.
26. Jones RD, Kerr DJ, Harnett AN, Rankin EM, Ray S and Kaye SB, A pilot study of quinidine and epirubicin in the treatment of advanced breast cancer. *Br J Cancer* **62**: 133–135, 1990.
27. Keilhauer G, Emiling F, Raschack M, Gries J and Schlick E, The use of R-verapamil is superior to racemic verapamil in breaking multidrug resistance of malignant cells. *Proc Am Assoc Cancer Res* **30**: 503, 1989.
28. Bisset D, Kerr DJ, Cassidy J, Meredith P, Traugott U and Kaye SB, Phase 1 and pharmacokinetic study of D-verapamil and doxorubicin. *Br J Cancer* **64**: 1168–1171, 1991.
29. Yin M-b, Bankusli I and Rustum YM, Mechanisms of the *in vitro* resistance to adriamycin and modulation by calcium channel blockers in mice. *Cancer Res* **49**: 4729–4733, 1989.
30. Mross K, New anthracycline derivatives: What for? *Eur J Cancer* **27**: 1542–1544, 1991.
31. Wishart GC, Plumb JA, Morrison JG, Hamilton TG and Kaye SB, Adequate tumour quinidine levels for multidrug resistance modulation can be achieved *in vivo*. *Eur J Cancer* **28**: 28–31, 1992.