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THE ACTIVITY OF VERAPAMIL AS A RESISTANCE MODIFIER *IN VITRO* IN DRUG RESISTANT HUMAN TUMOUR CELL LINES IS NOT STEREOSPECIFIC

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Abstract—The L-isomer of verapamil is a more potent calcium antagonist than the D-isomer. We have examined the two stereoisomers of verapamil for their ability to increase the chemosensitivity *in vitro* of three drug resistant cell lines (2780AD, MCF7/Adr^R and H69LX10). Neither racemic verapamil nor its individual isomers had any effect on the drug sensitivity of the parent cell lines (A2780, MCF7 and NCI-H69). Verapamil (6.6 μM) increased the sensitivity of all three resistant cell lines to Adriamycin® by 10–12-fold. This activity was concentration dependent and was maximal at 6–7 μM. The increase in sensitivity was only 2–3-fold at 2 μM, the maximum plasma concentration achieved in patients. Both the D- and L-isomers of verapamil alone at 6.6 μM were as effective as racemic verapamil and the D-isomer demonstrated the same concentration dependent activity as racemic verapamil. The total cellular Adriamycin® concentration of both 2780AD and MCF7/Adr^R was increased by two-fold in the presence of verapamil (6.6 μM). Both D- and L-verapamil alone increased the amount of drug accumulated to the same extent as racemic verapamil. These results indicate that the resistance modification activity of verapamil is not stereospecific. Use of D-verapamil alone in patients could increase the maximum tolerated plasma concentrations of verapamil and thus D-verapamil may be a more effective resistance modifier *in vivo* than racemic verapamil.

Induction of the multi-drug resistant phenotype in tumour cells *in vitro* is associated with the presence of a high molecular weight glycoprotein (P170) in the cell membrane. This protein is structurally related to a bacterial transport protein and is believed to act as an energy dependent drug efflux pump [1]. Through the action of this pump resistant cells maintain a lower intracellular drug concentration than drug sensitive cells incubated at the same external drug concentration [2]. Although first identified *in vitro*, there is now substantial evidence for the existence of P170 in human tumours suggesting that an active drug efflux pump is one mechanism of drug resistance *in vivo* [3].

A number of non-cytotoxic agents have been shown to reverse drug resistance *in vitro*, possibly by inhibition of the drug efflux mechanism [4]. Of these agents, verapamil has been shown to be active *in vitro* in a number of cell types and attempts have been made to use verapamil (which is a racemic mixture of the D- and L-isomers in equal proportions [5]) in combination with chemotherapy in cancer patients [6–8]. However, use in patients is accompanied by dose limiting hypotension and heart block [7]. As a result the plasma levels achieved in patients are between 3 and 12 times lower than the most effective concentration *in vitro* [6, 8, 9].

It is known that the L-isomer of verapamil is about 10 times more effective as a calcium antagonist than the D-isomer [10]. However, the two isomers are equipotent blockers of the fast inward current [11]. Since the effects of verapamil on drug resistance do not appear to be related to calcium antagonism [12] it is conceivable that the D-isomer alone would be a more suitable agent to use clinically, in view of the potential, though as yet undemonstrated, reduction in cardiovascular side-effects compared to the racemic mixture. We have thus investigated the activity of the two stereoisomers of verapamil as resistance modifiers.

MATERIALS AND METHODS

Materials. Racemic verapamil and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from the Sigma Chemical Co. (Poole, U.K.). The stereoisomers of verapamil were a gift from Knoll AG (6700 Ludwigshafen, F.R.G.). The D-isomer was 99% pure and the L-isomer 95% pure. Adriamycin® was obtained from Farmitalia (St Albans, U.K.) and [^{14}C]Adriamycin® (sp. act. 76.59 mCi/mmol) from Amersham International (Amersham, U.K.). Culture media were from Northumbria Biologicals (Cramlington, U.K.).

Cell lines. The human ovarian cell line A2780 and a drug resistant subline 2780AD were obtained from Dr R. F. Ozols (National Cancer Institute, Bethesda, U.S.A.). They were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing glutamine (2 mM) foetal calf serum (10%) and insulin (0.25 units/mL). The resistant line was grown in the presence of Adriamycin® (2 μM) and is known to express P170 [13].

A small-cell lung cancer cell line NCI-H69 was

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obtained from Dr D. Carney (National Cancer Institute, Bethesda, U.S.A.) and the Adriamycin® resistant subline H69LX10 was a gift from Dr P. Twentyman (M.R.C. Clinical Oncology and Radiotherapeutics Unit, Cambridge, U.K.). These lines grew in suspension and were maintained in RPMI 1640 medium containing glutamine (2 mM), foetal calf serum (10%) and, for H69LX10 only, Adriamycin® (1 μg/mL, 1.84 μM).

The human breast cancer cell line MCF7 and an Adriamycin® resistant subline MCF7/Adr® were obtained from Dr K. Cowan (National Cancer Institute, Bethesda, U.S.A.). They were grown in a mixture of Ham's F10 and Dulbecco's Modified Eagle Medium (50:50) containing glutamine (2 mM) and foetal calf serum (10%). The resistant subline was exposed to Adriamycin® (10 μM) for 24 hr every 6 weeks and has been shown to express P170 [14].

Drug resistant cell lines were grown in the absence of drug for 5 days prior to experiments.

Cytotoxicity assay. Drug sensitivity was determined by a tetrazolium based chemosensitivity assay as described previously [15]. Briefly, adherent cells were plated out at a density of 10^3 cells per well in 96 well, flat bottomed plates (Linbro from Flow Laboratories, 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, Watford, U.K.).

Exponentially growing non-adherent cells (NCI-H69 and H69LX10) were plated out at a density of 10^4 cells/well in 96 well round bottomed plates (Nunclon from Gibco, Paisley, U.K.) in 100 μL of medium. Drug was added immediately in 100 μL of medium at twice the final concentration. Plates were centrifuged (200 *g*, 5 min) prior to removal of medium and replacement with fresh medium on the next and following 4 days. Plates were then processed as for the adherent cells.

In experiments where modifiers were used, a serial dilution of eight concentrations of Adriamycin® 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 prior to addition to the wells. Four wells were used for each drug concentration and when modifiers were used the control wells were exposed to the modifier alone. The chemosensitivities of the drug resistant lines varied by a factor of three to four-fold between experiments. Therefore all experiments with modifiers included cells exposed to Adriamycin® alone to allow direct comparisons of drug sensitivities.

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 cells in the control untreated wells.

Drug accumulation. Cells were grown in 6 well plates (Nunclon) 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]Adriamycin® and Adriamycin® was 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 phosphate buffered saline (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 wells were transferred to scintillation vials, the wells washed with 0.5 mL 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 (Canberra Packard, Pangbourne, U.K.). Three wells were used for each time point and cell counts were determined from an additional three wells.

Detection of P-glycoprotein. Cytospin preparations of NCI-H69 and H69LX10 were fixed and stained as described elsewhere [16]. The monoclonal antibody C219 was a gift from Dr V. Ling and FITC-labelled sheep anti-mouse antibody was obtained from Scottish Antibody Production Unit (Law Hospital, Lanarkshire, U.K.).

RESULTS

Expression of P-glycoprotein

P-Glycoprotein was detected by immunostaining in the membrane of the drug resistant small-cell lung cancer cell line, H69LX10, but not in the parent cell line, H69, (Fig. 1).

Effects of verapamil on drug sensitivity

The sensitivity of the cell lines to Adriamycin® is shown in Table 1. The breast cell line MCF7/Adr^R is the most resistant and is about 750-fold more resistant than the parent line MCF7. The ovarian cell line 2780AD is about 7400-fold more resistant than A2780. The least resistant cell line is H69LX10 which is only about 60-fold more resistant than NCI-H69.

Also shown in Table 1 is the sensitivity to Adriamycin® when cells were exposed to the drug in the presence of either racemic verapamil (6.6 μM) or the individual stereoisomers of verapamil (6.6 μM). Neither verapamil nor the two stereoisomers had any significant effect on the chemosensitivity of the parent cell lines. However, verapamil increased the sensitivity to Adriamycin® of all three drug resistant cell lines by 10–12-fold. Furthermore, this effect was observed with both the D- and the L-isomers of verapamil.

Concentration dependent effect of varapamil

Figure 2 shows the sensitivity to Adriamycin® when cells are exposed to the drug in the presence of various concentrations of either racemic verapamil or the D-isomer of verapamil. For both 2780AD (Fig. 2A) and H69LX10 (Fig. 2B) there is a concentration dependent increase in drug sensitivity in the presence

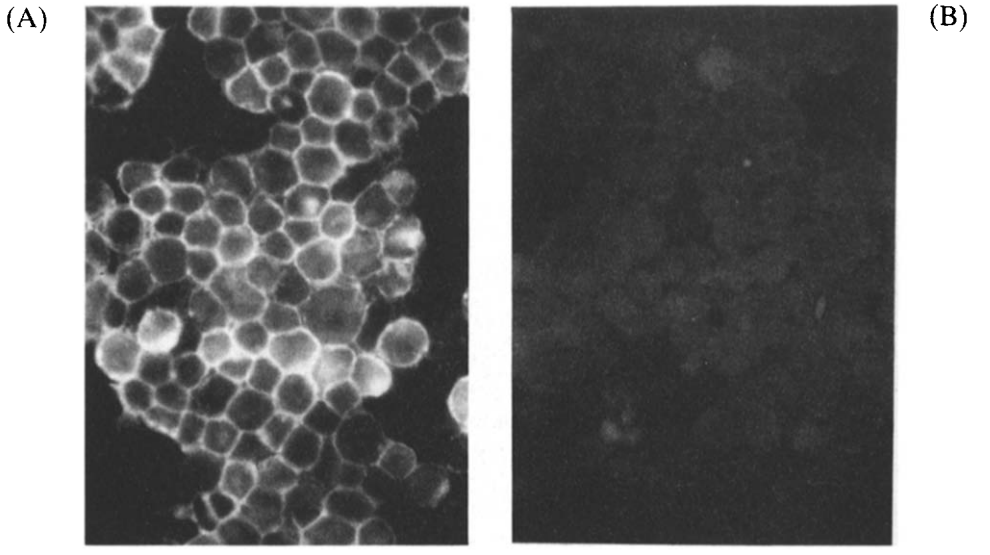

Fig. 1. Immunostaining of P-glycoprotein in the membrane of H69LX10 (A) but not NCI-H69 (B). Cells were stained with the monoclonal antibody C219 as the primary antibody and the second antibody was FITC-linked sheep anti-mouse.

Table 1. Sensitivity of the cell lines to Adriamycin® alone and in the presence of either racemic verapamil or one of the two stereoisomers of verapamil

	ID_{50} (nM)			
		Adriamycin® + Modifier (6.6 μM)		
Cell line	Adriamycin®	Verapamil	D-Verapamil	L-Verapamil
NCI-H69	45.0 ± 7.0	44.0 ± 6.2		
H69LX10	2680.0 ± 590.0	220.0 ± 50.0	270.0 ± 20.0	250.0 ± 40.0
A2780	1.0 ± 0.3	0.7 ± 0.3	0.6 ± 0.2	3.3 ± 0.7
2780AD	7430.0 ± 920.0	790.0 ± 30.0	580.0 ± 110.0	720.0 ± 40.0
MCF7	28.0 ± 12.0	24.3 ± 8.0	20.8 ± 1.3	14.7 ± 1.0
MCF7/ADR^R	21170.0 ± 3180.0	3670.0 ± 620.0	1750.0 ± 300.0	2170.0 ± 170.0

The ID_{50} is the drug concentration required to kill 50% of the cells and results are the mean ± SE of three observations.

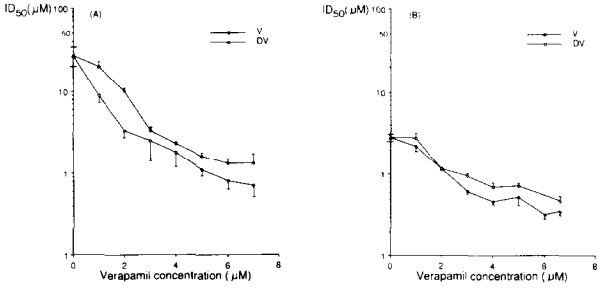

Fig. 2. Sensitivity of 2780AD (A) and H69LX10 (B) to Adriamycin® when exposed to drug in the presence of various concentrations of racemic verapamil (V) (●) or the D-isomer of verapamil (DV) (○). The ID_{50} is the drug concentration required to kill 50% of the cells and points are the mean ± SE of three observations.

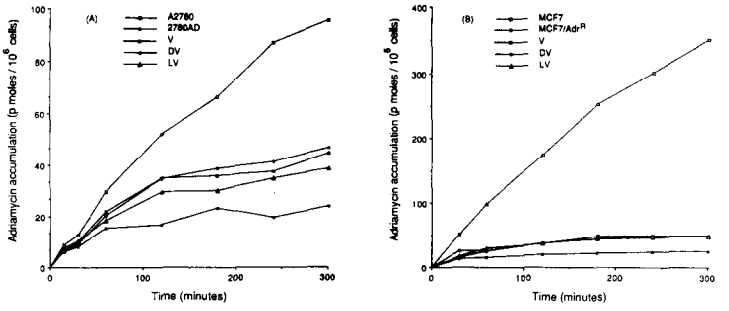

Fig. 3. Total cellular Adriamycin® accumulation following incubation of the cell lines (A) A2780 (□) and 2780AD (●); (B) MCF7 (□) and MCF7/Adr^R (●) in drug (1 μM). Also shown is the drug accumulation when the resistant lines were incubated in Adriamycin® (1 μM) in the presence of either racemic verapamil (■), D-verapamil (○) or L-verapamil (△) (all 6.6 μM). Points are the mean of three observations and error bars are too small to show.

of verapamil. This is true for both verapamil and the D-isomer of verapamil and there is no significant difference between the activities of the two agents at any given concentration. There is no significant difference between the ID_{50} at 6 and 7 μM ($P > 0.05$, Student's t-test) and this suggests that the activity of verapamil may be maximal at a concentration of 6–7 μM.

Effect of verapamil and D-*verapamil on drug accumulation*

Total cellular Adriamycin® content of the cell lines A2780 and 2780AD (A) and MCF7 and MCF7/Adr^R (B) following incubation for various times in Adriamycin® (1 μM) is shown in Fig. 3. After 5 hr the parent cell line A2780 had accumulated about four times as much Adriamycin® as the resistant cell line (2780AD). When incubated in the presence of verapamil (6.6 μM) the amount of Adriamycin® accumulated per cell was increased by about 80% in the resistant cell line (Fig. 3A). Both the D- and the L-isomers of verapamil produce a similar increase in drug accumulation.

The breast cell line MCF7 accumulated about 3–4 times as much Adriamycin® per cell as A2780 and about 14 times as much as the drug resistant cell line MCF7/Adr^R (Fig. 3B). In the presence of verapamil the amount of Adriamycin® accumulated by the resistant line was doubled but was still much less than that in the drug sensitive parent cell line (MCF7).

DISCUSSION

These results show clearly that the sensitivity of all three drug resistant cell lines to Adriamycin® is increased by 10–12-fold by verapamil (6.6 μM). Furthermore, both the D- and the L-isomers of verapamil used alone at this concentration are equally effective resistance modifiers.

The mechanism by which verapamil can increase the drug sensitivity of a cell line is not known. Several studies have shown that verapamil and a number of other compounds increase the intracellular drug concentration when compared with that of cells incubated with the cytotoxic drug alone [4, 17, 18]. We have shown that for the resistant cell line 2780AD the amount of drug accumulated for any given external concentration is only about 25% of that of the drug sensitive parent cell line. These results support previous observations [19]. When either of the drug resistant cell lines 2780AD and MCF7/Adr^R were incubated with cytotoxic drug in the presence of verapamil (6.6 μM) the amount of drug accumulated was almost doubled (Fig. 3). Incubation with either of the stereoisomers of verapamil at a concentration of 6.6 μM had a similar effect on drug accumulation. It is thought that drug resistant cells are able to maintain a decreased intracellular drug concentration due to the presence of an energy dependent drug efflux pump present in the cell membrane. Verapamil and other modifiers are thought to cause increased drug accumulation through inhibition of the efflux pump. The presence of the efflux pump is characterized by the presence of a high molecular weight glycoprotein (P170) in the cell membrane. All three drug resistant cell lines used in this study express this protein (Refs 13, 14 and Fig. 1).

Both the breast and ovarian drug resistant cell lines are an order of magnitude more resistant than the parent cell line in our hands than has been quoted elsewhere [14, 19]. This is not a reflection of the chemosensitivity assay used since we have shown that our assay gives results identical to those obtained by a standard clonogenic assay [15]. For the MCF7/Adr^R cell line the drug sensitivity quoted previously (4.8 μM) is less than the concentration used to select the cell line (10 μM). Since this line is only exposed to drug once in 6 weeks differences may relate to the time since the last drug selection. For the ovarian cell line the major difference relates to the drug sensitivity of the parent cell line A2780 which is about 10-fold more sensitive than the published value (12 nM c.f. Table 1). This may be due to differences in growth conditions since it is known that whilst the parent cell lines may be hormone and growth factor sensitive drug resistant sublines can lose these properties [20].

It is of note that although the three drug resistant cell lines have different drug sensitivities and different resistance factors the response to verapamil

by each line is of similar magnitude (Table 1, Fig. 3). In all cases drug sensitivity is increased by 10–12-fold and, at least for the two adherent cell lines (2780AD and MCF7/Adr^R), drug accumulation is doubled in the presence of verapamil. We do not have quantitative results on the relative amounts of P-glycoprotein in these cell lines. However, these results would suggest that there is a limit to the resistance modification achieved by verapamil. This is supported by our observations that the effect of verapamil on drug sensitivity is concentration dependent (Fig. 2) and that the resistance modification achieved at 7 μM verapamil is not significantly different from that achieved at 6 μM verapamil. These results would support the view that resistance to Adriamycin® is likely to be multifactorial, with the component relating to drug efflux which is reversible by verapamil forming one factor of varying importance. In selected tumour types however, modulation which may operate through this component might still prove to be clinically worthwhile.

Cornwell *et al.* [21] have shown that cytotoxic drugs bind to P170 and that bound drug can be displaced by various resistance modifiers including verapamil. These observations suggest that verapamil may prevent drug efflux by inhibiting binding of the cytotoxic drug to the pump protein. Our results with the two isomers of verapamil would suggest that this binding is not stereospecific. It is of interest, therefore, that both the D- and L-isomers of desmethoxyverapamil are able to displace the racemic mixture bound to membrane vesicles from drug resistant KB cells [21]. Both verapamil and desmethoxyverapamil had similar effects on drug accumulation in these cells.

P-Glycoprotein has been identified in a number of human tumours indicating that this mechanism of drug resistance, first identified *in vitro*, may be significant in clinical resistance [3, 8, 22–25]. This has led to a number of clinical studies in which standard chemotherapy is administered in combination with verapamil [6–8, 26]. A potential problem with this approach is the expression of P170 by normal human tissues including bone marrow [23, 27]. Although this might suggest that these tissue may become more sensitive to the cytotoxic drug in the presence of verapamil this does not appear to be true at least *in vitro* [28]. In all of the clinical studies the dose of verapamil used was limited by cardiovascular side-effects. The plasma concentrations achieved without toxicity were around 0.5–2 μM. Although there is an effect of verapamil on chemosensitivity at low concentrations the most marked effect is at 6–7 μM (Fig. 2). Whilst plasma levels may not necessarily reflect the concentration of verapamil within the tumour, it is probable that its cardioactivity will limit the potential activity of verapamil in the clinic.

However, the calcium antagonist and the cardiotoxic properties of verapamil are both stereoselective: the L-isomer is about 10-fold more active (in terms of effects on cardiac conduction) than the D-isomer [29–31]. Since we have shown that the D-isomer of verapamil is an effective resistance modifier, our results suggest that use of the D-isomer alone in patients might permit increased plasma concentrations to be obtained without cardiovascular side-effects and hence increase the potential for resistance modification in certain tumour types. Clinical trials of D-verapamil are now underway in our unit.

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