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The bisindolylmaleimide protein kinase C inhibitor, Ro 32-2241, reverses multidrug resistance in KB tumour cells

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Abstract Ro 32-2241 is a bisindolylmaleimide that selectively inhibits protein kinase C (PKC) as compared with other protein kinases. Experiments were carried out to examine its potential as a multidrug resistance-reversing agent. Ro 32-2241 inhibited efflux, and increased accumulation, of [^{3}H]-daunomycin in multidrug-resistant (MDR) KB-8-5 and KB-8-5-11 cells and had no effect on drug-sensitive KB-3-1 cells. Ro 32-2241 completely reversed the doxorubicin resistance of KB-8-5 and KB-8-5-11 cells, showing no effect on the sensitivity of drug-sensitive KB-3-1 cells. The potency of Ro 32-2241 was comparable with that of cyclosporin A and better than that of verapamil, known modulators of multidrug resistance. Ro 32-2241 also completely reversed the taxol resistance of KB-8-5 cells and partially reversed the resistance of KB-8-5-11 cells. Vinblastine resistance was also partially reversed. Mechanistic experiments were carried out to determine whether Ro 32-2241 interacted with P-glycoprotein (Pgp) directly. Increased efflux of [^{14}C]-Ro 32-2241 was seen with the more resistant KB-8-5-11 cells (although the percentage effluxed was very low as compared with [^{3}H]-daunomycin), suggesting that Ro 32-2241 can act as a substrate for Pgp. Direct interaction of Ro 32-2241 with Pgp was confirmed by demonstration that it inhibited binding of [^{3}H]-azidopine to Pgp in KB-8-5-11 membranes. In conclusion, Ro 32-2241, acting directly on Pgp (rather than, or in addition to, an effect on PKC), is effective in reducing or reversing resistance to doxorubicin, taxol and vinblastine in human tumour cells with a clinically relevant degree of MDR. However, results of in vivo experiments conducted to investigate the effects of Ro 32-2241 on resistance to doxorubicin suggest that it may not be possible to achieve sufficiently high levels of Ro 32-2241 in vivo to modulate MDR.

Key words Multidrug resistance · Reversal · P-glycoprotein · Protein kinase C inhibitors · Bisindolylmaleimides

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Introduction

Multidrug resistance (MDR), involving cross-resistance to chemotherapeutic drugs, is thought to be a significant obstacle in the successful treatment of several haematological malignancies [14, 24] and is likely to contribute to resistance in certain solid tumours [4, 34, 37]. It is frequently mediated by overexpression of the P-glycoprotein (Pgp), which functions as an ATP-dependent drug-efflux pump [18, 19]. A number of agents, including verapamil and cyclosporin A, bind to and inhibit Pgp to reverse MDR in vitro [4, 16, 35, 38]. However, to date, no effective resistance modifier has been useful in the clinic; these are generally dose-limited due to toxicity or unacceptable side effects [25].

Protein kinase C (PKC) activity levels are often altered in MDR tumour cells; several studies have demonstrated overexpression of PKCα in MDR cells [7, 10, 12, 21, 27, 28]. Other PKC subspecies may also be altered [7, 21]. Pgp is a substrate for PKC in vitro and in cells, and there is some evidence that increased phosphorylation of Pgp correlates with enhanced drug efflux [1, 9]. Staurosporine, a non-selective inhibitor of protein kinases, enhances drug accumulation, reduces drug efflux and reduces drug resistance in Pgp-expressing cells [8, 23, 26, 28, 32]. Other PKC inhibitors have also been shown to enhance drug accumulation and reduce drug efflux in Pgp-expressing cells [3, 8, 22, 29, 30, 31, 33, 39].

In this paper we explore the effects of Ro 32-2241, a highly selective inhibitor of PKC, on the functioning of

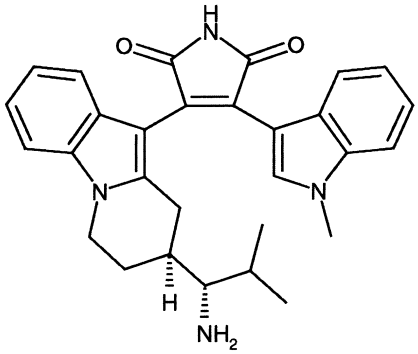

Fig. 1 Structure of Ro 32-2241

and interaction with Pgp. Ro 32-2241, shown in Fig. 1, is a bisindolylmaleimide that is related to a series of bisindolylmaleimide PKC inhibitors, including Ro 31-8220 and Ro 32-0432 [5, 6, 11]. Ro 32-2241 inhibits isolated (rat brain) PKC enzyme activity at an IC_{50} of 6 n*M*. It is highly selective for PKC relative to the other kinases studied; other serine/threonine protein kinases are inhibited at IC_{50} values in excess of 2 μ*M*, and src tyrosine kinases are inhibited at IC_{50} values in excess of 100 μ*M*. Ro 32-2241 is orally bioavailable and effective at inhibiting PKC-dependent processes in vivo.

These studies focused on well-characterised low-level MDR cell lines KB-8-5-11 and KB-8-5, derived from the human oral epidermoid carcinoma cell line KB-3-1 [2, 15], whose properties are summarised in Table 1. Low-level resistant members of the KB series were chosen for the work as they have the most clinically relevant degrees of drug resistance. The PKC activity has been shown to be elevated in KB-8-5-11 cells as compared with KB-3-1 cells [12]. The effect of Ro 32-2241 on Pgp activity and drug resistance in vitro and in vivo was studied and compared with the effects of cyclosporin A and verapamil, known resistance modifiers.

Materials and methods

Materials

Colchicine, daunomycin, doxorubicin (Adriamycin), verapamil, vinblastine, etoposide and paclitaxel (taxol) were obtained from Sigma Chemical (Poole, Dorset, UK). Cyclosporin A was supplied by Research Biochemicals International (Nantwich, Mass., USA). Ro 32-2241 (3-[8(S)-(1(S)-amino-2-methylpropyl)-6,7,8,9-tetrahydro = pyrido[1,2-a]indol-10-yl]-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione hydrochloride) and [^{14}C]-Ro 32-2241 (3-[8(S)-(1(S)-amino-2-methylpropyl)-6,7,8,9-tetrahydro = pyrido[1,2-a]indol-10-yl]-4-(1-methyl-3-indolyl)-1H-pyrrole[5-C14]-2,5-dione hydrochloride) were synthesised at the Department of Chemistry, Roche Discovery Welwyn (Welwyn Garden City, Herts, UK). [^{3}H(G)]-Daunomycin (1–5 Ci/mmol) was obtained from NEN Research Products (Du Pont Ltd, Stevenage, Herts, UK). [^{3}H]-Azidopine (40–60 Ci/mmol) and [methyl-^{3}H]-thymidine (5 Ci/mmol) were provided by Amersham International plc (Little Chalfont, Bucks, UK). Trypsin/ethylenediaminetetra acetic acid (EDTA, 10x) and all cell culture reagents were obtained from Gibco BRL (Life Technologies Ltd, Paisley, Scotland). All other reagents were of the highest available grade and were supplied by either Sigma or BDH.

Cell culture

The KB human carcinoma cell lines KB-3-1, KB-8-5 and KB-8-5-11 [2] were kindly provided by Dr. M. Gottesman (National Cancer Institute, Bethesda, Md., USA). The cells were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and penicillin-streptomycin at 37 °C in 5% CO_2/95% air. The cell lines were maintained as follows in the appropriate concentration of drug in which they were selected: no addition for drug-sensitive KB-3-1 cells; 10 ng colchicine/ml for KB-8-5 cells; and 100 ng colchicine/ml for KB-8-5-11 cells [2]. Cells were maintained in 75-cm^2 flasks and passaged into either 6- or 96-well plates for subsequent experiments.

Drug efflux and accumulation studies

Efflux of [^{3}H]-daunomycin is used as an indicator of Pgp activity, and accumulation can also be measured in the same experiments [13]. Cells were passaged into 6-well plates in drug-free medium and then maintained in culture overnight to achieve approx. 80% confluency before their use in the experiments. The medium was then removed, and the cells were washed twice in DMEM and incubated for 1 h at 37 °C in DMEM (1 ml) containing daunomycin (10 μ*M*), [^{3}H]-daunomycin (0.5 μCi) and Ro 32-2241 (as required; similar experiments were carried out where the cells were preincubated for 48 h with Ro 32-2241, and daunomycin plus [^{3}H]-daunomycin were added for the last hour). The monolayers were then washed once with DMEM, and fresh DMEM (1 ml) containing Ro 32-2241 (as in the previous addition) was added. Incubations were continued at 37 °C, and aliquots of medium (100 μl) were removed at the time points required for efflux measurements (10, 30 and 60 min). These aliquots were immediately centrifuged to remove any residual cells, and the supernatant was taken for liquid scintillation counting. At the end of the efflux incubations the cell monolayers were washed twice in ice-cold phosphate-buffered saline (PBS) and then solubilised in 10% Triton-X-100, and an aliquot was taken for liquid scintillation counting. This final sample gives the amount of [^{3}H]-daunomycin remaining in the cells, from which the initial total cell content (accumulation of [^{3}H]-daunomycin after 1 or 48 h of incubation) can be calculated by addition of the former to the total amount

Table 1 Characteristics of the KB cell lines used in this study[a]

Cell line	Selection	Relative resistance to			MDR1 mRNA	Pgp (ng/ml)
		Colchicine	Adriamycin	Vinblastine		
KB-3-1	Parent	1	1	1	1	Not detectable
KB-8-5	Colchicine, 10 ng/ml	4	3	6	42	Detectable at low level[b]
KB-8-5-11	Colchicine, 100 ng/ml	40	23	51	240	143

[a] Data from Richert et al. [29]
[b] Drew et al. (unpublished observation)

effluxed. Efflux is expressed as a percentage of the initial total cell content of [^{3}H]-daunomycin. Incubations were carried out in duplicate within each experiment, and experiments were repeated at least three times.

A similar method was used to measure the efflux of [^{14}C]-Ro 32-2241. Cells were cultured as described above, then incubated for 1 h with 10 μ*M* [^{14}C]-Ro 32-2241. After washing and addition of fresh DMEM, samples were taken every 10 min for measurement of efflux as described above.

Binding of [^{3}H]-azidopine to Pgp

This was based on the method described by Miyamoto et al. [26]. Cells were grown to 80–90% confluency in 75-cm^2 flasks, washed with PBS and harvested in solution A [10 m*M* TRIS-HCl (pH 7.4), 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.02 m*M* phenylmethylsulfonyl fluoride (PMSF)]. Following homogenisation with 25–30 strokes using a Dounce homogeniser the cell homogenate was overlayed onto a 1.5 *M* sucrose cushion and centrifuged for 1 h at 18,000 *g*. The membrane interface was then removed, diluted in solution A and centrifuged for 1 h at 100,000 *g*. The membrane pellet obtained was resuspended in 10 m*M* TRIS-HCl (pH 7.4) containing 250 m*M* sucrose and the protein content, determined by the Pierce BCA method. Next, 50 μg membrane protein was incubated with the test compound in 10 m*M* TRIS-HCl (pH 7.4) containing 1 μCi [^{3}H]-azidopine in a final volume of 50 μl at room temperature in darkness for 30 min. The samples were then exposed to long-wave UV irradiation on ice for 20 min. Samples were diluted in sample buffer(1:6), heated to 70 °C for 2 min and analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% polyacrylamide). [^{3}H]-Azidopine binding to Pgp was detected by fluorography and quantitated by densitometry.

Inhibition of proliferation of KB cells

DNA synthesis was measured as a representation of cell proliferation [17]. KB cells were passaged into 96-well plates (at approx. 3×10^3 cells/well) in drug-free DMEM. They were maintained at 37 °C under 5% CO_2/95% air for 4–6 h and then incubated for 48 h with Ro 32-2241 and/or various other drugs (see results) as required. All drugs were made up as stock solutions in dimethylsulfoxide (DMSO); the final concentration of DMSO (also included in the control wells) was consistent and did not exceed 0.1%. [^{3}H]-Thymidine (0.5 μCi/well) was added for the last 18 h. The wells were then washed, the adherent cells were removed by addition of trypsin/EDTA (3x; 50 μl), and the cells were harvested onto glass-fibre filters. The filters were impregnated with solid scintillant, and each of the 96 positions on the filter were counted. Incubations were carried out in triplicate within each experiment, and experiments were repeated at least three times.

In vivo studies

Female MF1/*nu* mice aged 6–8 weeks were obtained from Harlan UK Ltd (Shaw's Farm, Oxon). KB cells were suspended (at 2×10^7/ml) in sterile PBS. Mice were implanted with 2×10^6 cells by s.c. injection into the flank. Each animal received two implants, drug-sensitive (KB-3-1) cells on the left flank and resistant (KB-8-5 or KB-8-5-11) cells on the right flank. Animals were dosed beginning on day 3 of implantation (see Results for individual details). All experiments had 10 animals in each treatment group. Ro 32-2241 was formulated in 10% succinylated gelatin. Doxorubicin was formulated in PBS. Where appropriate, animals were dosed with Ro 32-2241 at 3 h prior to treatment with doxorubicin. Animals were monitored daily for signs of tumour growth. At the end of the dosing period (day 18 post-implantation), animals were killed and the tumours were excised and weighed.

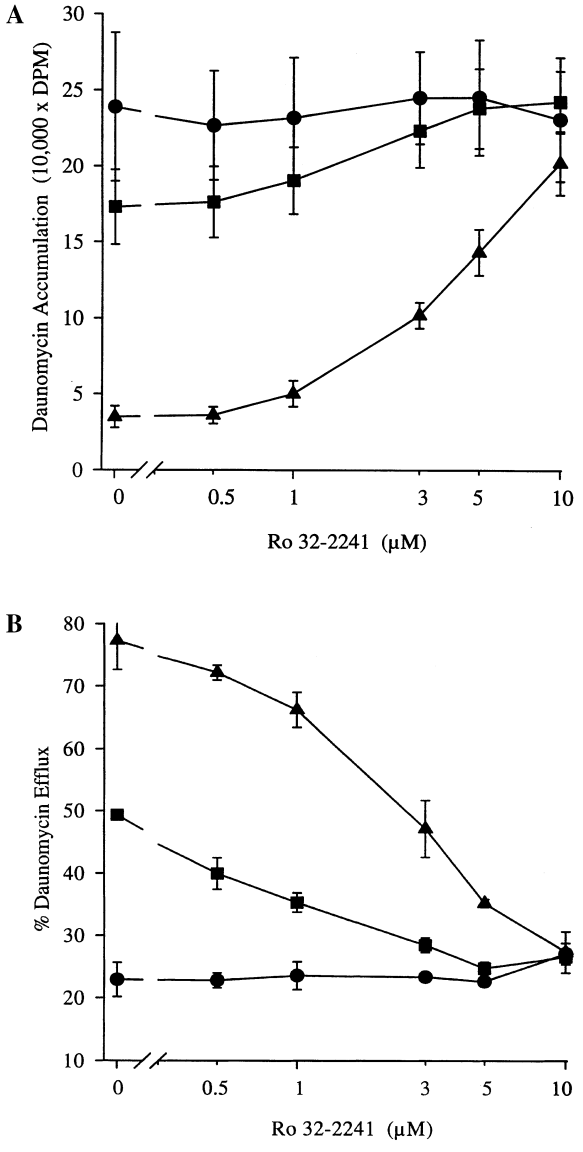

Fig. 2A,B Effect of Ro 32-2241 on the accumulation and efflux of [^{3}H]-daunomycin from KB tumour cells. **A** Accumulation of [^{3}H]-daunomycin in the cells was measured after 60 min of incubation and **B** efflux of [^{3}H]-daunomycin from the cells was measured after a further 30 min of incubation. Results are shown as obtained from KB-3-1 (●), KB-8-5 (■) and KB-8-5-11 (▲) cells and represent mean values ± SEM for 3 separate experiments. Accumulation is expressed as the dpm of [^{3}H]-daunomycin observed in the cells after 60 min of incubation. Efflux, after a further 30 min, is expressed as a percentage of the accumulated [^{3}H]-daunomycin

Results

Effect of Ro 32-2241 on Pgp activity: efflux and accumulation of [^{3}H]-daunomycin

Figure 2A shows the effect of Ro 32-2241 on accumulation of [^{3}H]-daunomycin in the three KB cell lines. There is a high level of [^{3}H]-daunomycin accumulation in the drug-sensitive KB-3-1 cells that is unaffected by Ro 32-2241. There is a very low level of accumulation in

the KB-8-5-11 cells, and Ro 32-2241 dose-dependently restores the level of accumulation back to that of the drug-sensitive KB-3-1 cells. The response of the KB-8-5 cells is intermediate but nonetheless restored by Ro 32-2241. Restoration of [^{3}H]-daunomycin accumulation in the resistant cells to the level seen in the drug-sensitive cells suggests that Ro 32-2241 is inhibiting efflux of [^{3}H]-daunomycin by Pgp.

For confirmation that Ro 32-2241 was having a direct effect on the efflux of drug rather than an effect on its uptake, experiments were carried out to measure efflux directly. Efflux and accumulation of [^{3}H]-daunomycin were measured in the same experiments. Figure 2B shows the effect of Ro 32-2241 on [^{3}H]-daunomycin efflux from KB-3-1, KB-8-5 and KB-8-5-11 cells as measured after 30 min. Essentially similar results were obtained after 10 and 60 min (data not shown). There is a low basal level of Pgp-independent [^{3}H]-daunomycin efflux from the KB-3-1 cells that is unaffected by Ro 32-2241. Efflux is highest from the KB-8-5-11 cells, as would be expected since they are the more resistant cells and express higher levels of Pgp. Efflux from the KB-8-5 and KB-8-5-11 cells is dose-dependently inhibited by Ro 32-2241 at IC_{50} values (mean ± SEM, $n = 3$) of 1.01 ± 0.17 and 2.91 ± 0.42 μM, respectively. Efflux is completely inhibited at the higher concentrations of Ro 32-2241 down to the same level as that seen with the drug-sensitive KB-3-1 cells. The results shown in Fig. 2 derive from experiments where the cells were preincubated for 1 h with Ro 32-2241. Since all experiments measuring the proliferation of KB cells require 48 h of preincubation with Ro 32-2241, the efflux experiments were also carried out with a 48-h period of preincubation with Ro 32-2241. The IC_{50} for Ro 32-2241 is similar after either 1 h or 48 h of preincubations (data not shown). In each case the KB-8-5-11 cells (IC_{50} approx. 1 μM) are less sensitive than the KB-8-5 cells (IC_{50} approx. 3 μM).

For further investigation of this, experiments were then carried out to see whether these cells could pump out Ro 32-2241. The protocol was essentially similar to that used for [^{3}H]-daunomycin efflux, except that the cells were labeled with [^{14}C]-Ro 32-2241 (10 μM). Figure 3A shows [^{14}C]-Ro 32-2241 efflux from KB-3-1, KB-8-5 and KB-8-5-11 cells; the percentage of [^{14}C]-Ro 32-2241 efflux is shown on the same scale as that of [^{3}H]-daunomycin efflux. There is a very low Pgp-independent basal efflux of [^{14}C]-Ro 32-2241 from the KB-3-1 cells, and no additional loss from the KB-8-5 cells is visible. There is a small increase in efflux of [^{14}C]-Ro 32-2241 from the KB-8-5-11 cells, consistent with the observation that KB-8-5-11 cells are less sensitive to Ro 32-2241 for inhibition of [^{3}H]-daunomycin efflux.

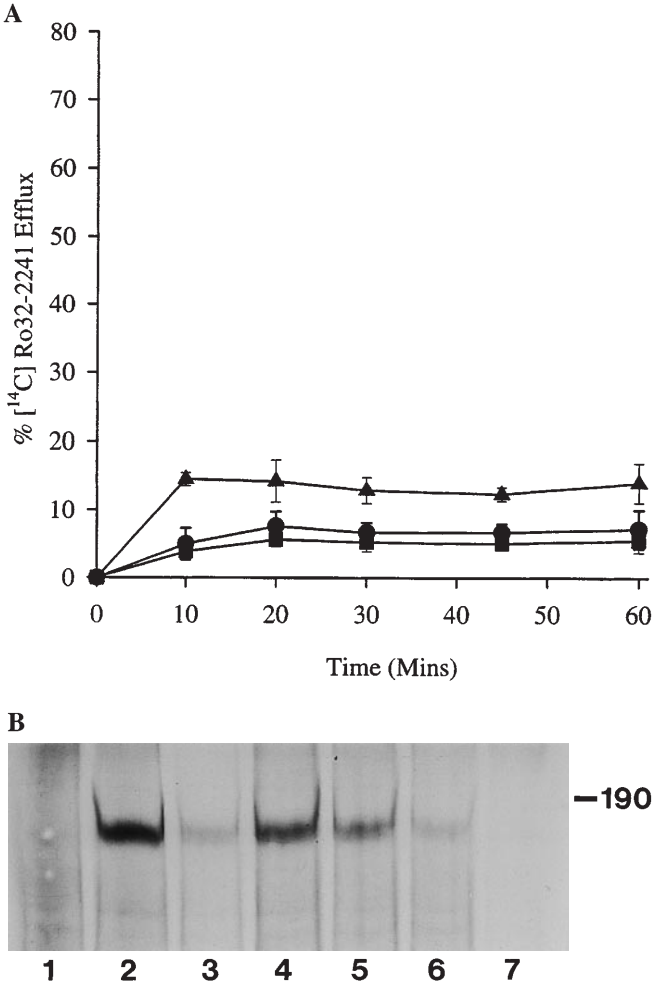

Fig. 3A,B Interaction of Ro 32-2241 with Pgp. **A** Efflux of [^{14}C]-Ro 32-2241 (10 μM) from KB-3-1 (●), KB-8-5 (■) and KB-8-5-11 (▲) cells. This graph is drawn on the same scale as Fig. 2, representing [^{3}H]-daunomycin efflux. Results are shown as mean values ± ranges for 2 separate experiments. Efflux, at the times shown, is expressed as a percentage of the accumulated [^{14}C]-Ro 32-2241. **B** Inhibition of [^{3}H]-azidopine binding to Pgp in KB-8-5-11 cell membranes. [^{3}H]-Azidopine labeling of Pgp was carried out for KB-3-1 cell membranes in the presence of DMSO (*lane 1*) and for KB-8-5-11 cell membranes in the presence of DMSO (*lane 2*), vinblastine at 10 μM (*lane 3*) and Ro 32-2241 at 100 nM (*lane 4*) 1 μM (*lane 5*), 10 μM (*lane 6*) and 100 μM (*lane 7*). Scanning data from this figure are shown in Table 2

Effect of Ro 32-2241 on binding of [^{3}H]-azidopine

Experiments were carried out to examine the possibility that Ro 32-2241 could bind to Pgp. Well-known Pgp substrates such as vinblastine are known to bind directly to Pgp; this can be demonstrated by examination of the inhibition of [^{3}H]-azidopine binding to Pgp [20, 26]. Figure 3B and Table 2 (data derived from Fig. 3B) show that Ro 32-2241 inhibits [^{3}H]-azidopine binding to Pgp in membranes from KB-8-5-11 cells. The effect of Ro 32-2241 is concentration-dependent, with 90% inhibition occurring at 10 μM; this degree of inhibition is comparable with that seen with 10 μM vinblastine. There was little detectable binding of [^{3}H]-azidopine to the drug-sensitive KB-3-1 membranes, which do not express Pgp (lane 1). It is therefore clear that Ro 32-2241 can interact directly with Pgp.

Table 2 Binding of [^{3}H]-azidopine to Pgp in membranes of KB cells: scanning data from Fig. 3B

Cell type	Treatment	Area	Percent maximum
KB-3-1	Control	0.073	4.5
KB-8-5-11	Control	1.608	100
KB-8-5-11	Vinblastine (10 μM)	0.197	12.3
KB-8-5-11	Ro 32-2241 (100 nM)	1.034	64.3
KB-8-5-11	Ro 32-2241 (1 μM)	0.528	32.8
KB-8-5-11	Ro 32-2241 (10 μM)	0.16	10.0

Effect of Ro 32-2241 alone on [^{3}H]-thymidine incorporation into KB cells

Figure 4 shows the effect of Ro 32-2241 on [^{3}H]-thymidine incorporation into KB cells; normalised data combined from 5 experiments are expressed as percentages of control values obtained in the absence of Ro 32-2241. Ro 32-2241 dose-dependently inhibits proliferation at IC_{50} values (mean ± SEM, $n = 5$) of 1.29 ± 0.18 μM on the KB-3-1 cells, 1.20 ± 0.14 μM on the KB-8-5 cells and 3.01 ± 0.63 μM on the KB-8-5-11 cells.

Effect of Ro 32-2241 on sensitivity of KB cells to chemotherapeutic agents

[^{3}H]-Thymidine incorporation was measured as described above. The cells were preincubated for 48 h with various concentrations of Ro 32-2241 and a full range of concentrations of chemotherapeutic agent. The aim of the experiments was to see if Ro 32-2241 modulated the sensitivity of the cells to chemotherapeutic agents. The concentrations of Ro 32-2241 chosen span the IC_{50} spectrum for inhibition of Pgp activity. KB-8-5-11 cells are less sensitive to Ro 32-2241; hence, 2-fold higher concentrations of Ro 32-2241 were used with these cells. Since Ro 32-2241 itself inhibits proliferation (see Fig. 4), the results are expressed as a percentage of the control counts per minute obtained in the absence of chemotherapeutic agents. Dose curves for chemotherapeutic agents were then constructed in the presence of various concentrations of Ro 32-2241. The resistance modification index is calculated as (*IC_{50} of cytotoxic drug + modifying agent*)/(*IC_{50} of cytotoxic drug alone*).

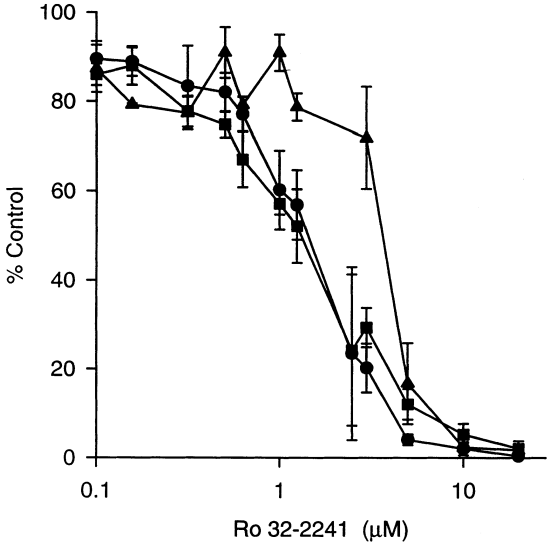

Fig. 4 Effect of Ro 32-2241 alone on [^{3}H]-thymidine incorporation into KB cells. After 48 h of incubation with Ro 32-2241, [^{3}H]-thymidine incorporation into KB-3-1 (●), KB-8-5 (■) and KB-8-5-11 (▲) cells was measured. Normalised data combined from 5 experiments (mean values ± SEM) are expressed as percentages of control values obtained in the absence of Ro 32-2241. The actual [^{3}H]-thymidine cpm (mean values ± SEM, $n = 5$) incorporated into controls were as follows: KB-3-1, 51,631 ± 8,259; KB-8-5, 50,793 ± 5,809; and KB-8-5-11, 68,257 ± 9,785

Table 3 shows the effect of Ro 32-2241 on the inhibition of [^{3}H]-thymidine incorporation into KB-3-1, KB-8-5 and KB-8-5-11 cells by doxorubicin. The IC_{50} values and resistance modification indices (RMI) from 15 separate experiments are summarised. It is clear that Ro 32-2241 has no effect on the doxorubicin sensitivity of the drug-sensitive KB-3-1 cells, even at the highest concentration of Ro 32-2241, which itself inhibits proliferation. The doxorubicin resistance of KB-8-5 cells is completely reversed by 1 μM Ro 32-2241, and the resistance of KB-8-5-11 cells is almost completely reversed by 4 μM Ro 32-2241.

Cyclosporin A reversed the resistance of the KB-8-5 cells at 0.5 μM, and at 4 μM it reversed that of the KB-8-5-11 cells; these concentrations are comparable with the concentrations of Ro 32-2241 required in similar experiments. Verapamil reversed the resistance of the KB-8-5 cells at 1 μM, and at 10 μM it reversed that of the KB-8-5-11 cells; this capacity for MDR reversal appears to be less potent than that of Ro 32-2241 (Table 3).

Table 3 also summarises the IC_{50} values and resistance modification indices noted for the effects of Ro 32-2241 on sensitivity to vinblastine and taxol. Ro 32-2241 increased the sensitivity of both KB-8-5 (110-fold resistant) and KB-8-5-11 cells (450-fold resistant) to vinblastine, although the highest concentration of Ro 32-2241 tested did not restore sensitivity to that of the drug-sensitive KB-3-1 cells. The taxol resistance of the KB-8-5 cells (17-fold resistant) is completely reversed by 1–2 μM Ro 32-2241, and the resistance of KB-8-5-11 cells (146-fold resistant) is reduced, but not completely reversed, by 4 μM Ro 32-2241.

Effect of Ro 32-2241 on MDR in vivo

A preliminary experiment (Fig. 5A) showed that a doxorubicin dose of 4 mg/kg i.v. 2x weekly was a suitable dose of doxorubicin alone to give inhibition of growth of the drug-sensitive KB-3-1 cells along with a lesser effect on the resistant KB-8-5 cells when the cells were grown as s.c. implants in nude mice. Preliminary experiments also showed that 100 mg/kg Ro 32-2241

Table 3 Modification of doxorubicin, vinblastine, taxol and etoposide resistance by Ro 32-2241 and of doxorubicin resistance by verapamil and cyclosporin A. IC_{50} values were based on [^{3}H]-thymidine incorporation as described in Materials and methods. The resistance modification index (*RMI*) is calculated as (IC_{50} of cytotoxic drug + modulator/ IC_{50} of cytotoxic drug alone)

Modulator	Modulator concentration (μM)	Anticancer drug	KB-3-1		KB-8-5		KB-8-5-11	
			IC_{50} (nM)	RMI	IC_{50} (nM)	RMI	IC_{50} (nM)	RMI
Ro 32-2241 ($n = 15$)	0	Doxorubicin	9.1	1	28	1	341	1
	0.5		8.4	0.92	12	0.43		
	1		6.5	0.71	6.5	0.23	97	0.28
	2		7.9	0.87	6.6	0.24	33	0.10
	4						11	0.03
Verapamil ($n = 7$)	0	Doxorubicin	9.1	1	30	1	198	1
	1		2.2	0.24	3.8	0.13	69	0.35
	2		5	0.55	3.8	0.13	21	0.11
	5		6.1	0.67	9	0.30	16	0.08
	10		7.5	0.82	6.7	0.22	7.4	0.04
	20		6	0.66	11	0.37	12	0.06
Cyclosporin A ($n = 4$)	0	Doxorubicin	4.2	1	16	1	120	1
	0.5		4.9	1.17	2.9	0.18	49	0.41
	1		2.5	0.60	2.5	0.16	11	0.09
	2		2.5	0.60	2.7	0.17	7	0.06
	4		5.2	1.24	2.6	0.16	3.6	0.03
Ro 32-2241 ($n = 4$)	0	Vinblastine	0.07	1	7.7	1	32	1
	0.5		0.09	1.29	2.5	0.32		
	1		0.1	1.43	1.3	0.17	13	0.41
	2		0.19	2.71	0.6	0.08	6.2	0.19
	4						2.1	0.07
Ro 32-2241 ($n = 6$)	0	Taxol	1	1	17	1	146	1
	0.5		0.5	0.50	3.3	0.19		
	1		0.7	0.70	1.2	0.07	61	0.42
	2		1.5	1.50	0.9	0.05	64	0.44
	4						26	0.18

given 5 times/week over a 4-week period was the maximum tolerated dose. This produced plasma levels of 3,988 ± 171 ng/ml ($n = 5$), a range of 5–10 μM, which is above the concentration required to inhibit Pgp and reverse resistance in vitro. The effect of combination of Ro 32-2241 (100 mg/kg per day p.o.) with 4 mg/kg doxorubicin (i.v. 2x weekly) was then examined. Animals were implanted with KB-3-1 cells in the left flank and either KB-8-5 or KB-8-5-11 cells in the right flank (Fig. 5B). Where appropriate, Ro 32-2241 was given 3 h before doxorubicin. The growth of all three cell lines was unaffected by a 100-mg/kg daily oral dose of Ro 32-2241 alone. The growth of all three lines was inhibited to some extent by twice-weekly dosing of 4 mg/kg i.v. doxorubicin alone, although (as expected) the drug-sensitive KB-3-1 cells appeared to be the most sensitive. Ro 32-2241 did not show a significant effect on the sensitivity of any of the cell lines (resistant or drug-sensitive) to doxorubicin. However, it is noteworthy that in this experiment, 4 mg/kg doxorubicin given without Ro 32-2241 reduced the growth of the KB-8-5 and KB-8-5-11 cells close to that of the KB-3-1 cells, leaving rather little scope for reversal of resistance. The difference observed between KB-3-1 and KB-8-5 cells following doxorubicin treatment without Ro 32-2241 was smaller than that seen in the preliminary experiment (Fig. 5A).

Discussion

The present study shows that Ro 32-2241, a bisindolylmaleimide selective inhibitor of PKC, can completely inhibit the efflux of [^{3}H]-daunomycin from Pgp-expressing KB-8-5 and KB-8-5-11 cells, resulting in increased daunomycin accumulation. Ro 32-2241 was more potent than verapamil and showed potency similar to that of cyclosporin A in reversing resistance in these cells.

It may be that Ro 32-2241 reverses resistance via inhibition of PKC, consistent with previous observations that phosphorylation of Pgp by PKC correlates with enhanced drug efflux [9]. However, several studies have shown that there is no correlation between inhibition of protein kinase activity in vitro and effects on drug accumulation in MDR cells [36, 40]. Since Ro 32-2241 inhibits PKC in an ATP-competitive manner [11] and binding of ATP to Pgp is required for drug efflux, the possibility that Ro 32-2241 might interact directly with Pgp was considered. Indeed, although other PKC inhibitors – including staurosporine, calphostin C and NPC 15437 – are not all ATP-competitive, they have been shown to inhibit photoaffinity labeling of this protein [3, 32, 33, 40]. Evidence was obtained suggesting that Ro 32-2241 is a substrate for Pgp. [^{14}C]-Ro

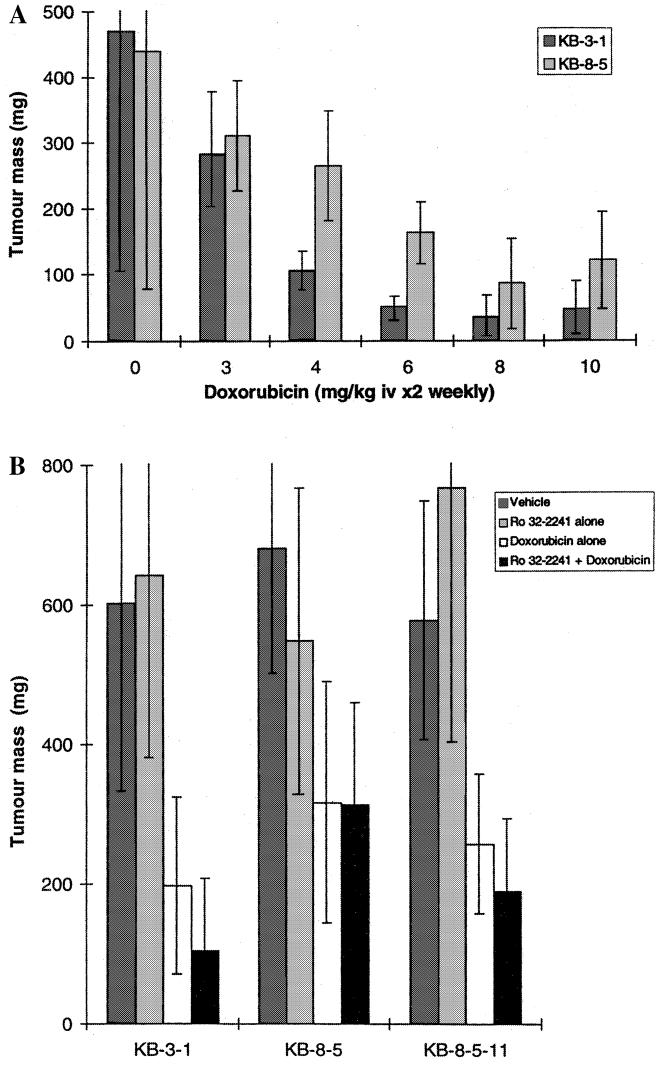

Fig. 5A,B Growth of KB cells in MF1/nu mice. **A** Effects of doxorubicin dosed i.v. twice weekly. Results (mass of tumour) are shown as mean values ± SEM recorded for groups of 10 animals, except at higher doses of doxorubicin, where animals were lost due to its toxicity (at 6 mg/kg, $n = 9$; at 8 mg/kg, $n = 6$; at 10 mg/kg, $n = 2$). **B** Effects of Ro 32-2241 (100 mg/kg per day p.o.) and doxorubicin (4 mg/kg i.v. 2x weekly). Results are shown as mean values ± SEM recorded for groups of 20 animals with KB-3-1 cells and for 10 animals with KB-8-5 and KB-8-5-11 cells, except where animals were lost in the last 3 days in the Ro 32-2241 + doxorubicin treatments, where $n = 7$ for KB-8-5 cells and $n = 9$ for the KB-8-5-11 cells

32-2241 efflux from the KB-8-5 cells appeared to be no different from the very small baseline leak seen from the drug-sensitive KB-3-1 cells, whereas there was a higher level of efflux from the KB-8-5-11 cells. Ro 32-2241 clearly inhibited binding of [^{3}H]-azidopine to Pgp, suggesting that Ro 32-2241 may interact with Pgp. Thus, the effects of Ro 32-2241, like the effects of other PKC inhibitors, may be a result of direct interaction with Pgp, inhibition of PKC, or some combination of both mechanisms.

Regardless of the mode of action of Ro 32-2241, it is highly effective at inhibiting drug efflux from MDR cells in vitro. Ro 32-2241 could completely restore the sensitivity of KB-8-5 and KB-8-5-11 cells to doxorubicin. The resistance of the KB 8-5 cells to taxol was completely reversed by Ro 32-2241, and that of the KB-8-5-11 cells was partially reversed.

The effects of Ro 32-2241 on resistant cells in vivo were also examined but did not demonstrate a significant increase in the effects of doxorubicin on tumour mass under conditions where the plasma concentrations of Ro 32-2241 should be capable of reversing Pgp activity as based on in vitro studies. It may be that there was insufficient localisation of Ro 32-2241 to the tumour mass. Alternatively, it may be appropriate to examine the effect of Ro 32-2241 in combination with lower concentrations of doxorubicin; we note that the KB-8-5-11 tumour mass had been reduced by doxorubicin to a level close to that achieved for the KB-3-1 tumours by doxorubicin alone, leaving only limited scope for potentiation by Ro 32-2241.

In conclusion, Ro 32-2241, acting directly on Pgp and/or via an effect on PKC, is effective in reducing or reversing resistance to doxorubicin, taxol and vinblastine in human tumour cells with a clinically relevant degree of MDR. The in vitro potency of Ro 32-2241 is comparable with that of cyclosporin A and better than that of verapamil. However, an initial experiment did not provide evidence for modulation of MDR in vivo by Ro 32-2241.

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