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KT-5720 Reverses Multidrug Resistance in Variant S49 Mouse Lymphoma Cells Transduced With the Human *MDR1* cDNA and in Human Multidrug-resistant Carcinoma Cells

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T-25-Adh cells, cell variants derived from S49 mouse lymphoma, were transduced with a retrovirus containing the human *MDR1* cDNA. The resultant cells (HU-1) are cross-resistant to colchicine, doxorubicin, vinblastine and actinomycin D, and their resistance to colchicine is reversed by verapamil. HU-1 cells were used to screen several protein kinase modulators for their ability to reverse multidrug resistance. Among the tested indole carbazole (K-252a) family of protein kinase inhibitors, only the antibiotic alkaloid KT-5720 (9-n-hexyl derivative of K-252a) could overcome the multidrug resistance of HU-1 cells and KB-V1 human carcinoma cells. Since other protein kinase A, C and G modulators did not reverse multidrug resistance in the tested multidrug-resistant cells, the chemosensitising activity of KT-5720 on these cells is apparently independent of its kinase inhibitory effects. Since KT-5720 fully reversed multidrug resistance at non-toxic concentrations, it might be a candidate for clinical chemosensitisation in combination chemotherapy.

Key words: chemosensitisers, K-252a derivatives, KT-5720, *MDR1*, multidrug resistance, P-glycoprotein, protein kinases, protein kinase inhibitors
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INTRODUCTION

CLINICAL RESISTANCE to chemotherapeutic drugs is a major obstacle in cancer therapy. One form of drug resistance, termed multidrug resistance (MDR), is defined as the capability of malignant cells subjected to a single cytotoxic agent to develop resistance to many structurally and functionally unrelated drugs

[1, 2], due to enhanced efflux transport of cytotoxic drugs [3–7]. The most consistent alteration found in multidrug-resistant cells is expression of a high molecular weight, cell surface glycoprotein (P-glycoprotein) and the concurrent reduction in the accumulation and/or retention of cytotoxic drugs. The genetic, biochemical and functional properties of P-glycoprotein have been intensively investigated and characterised (for reviews, see [3–7]). P-glycoprotein is expressed in some normal tissues and in many tumours where its physiological role is unknown [7]. A study of over 400 tumours has shown a consistent association of *MDR1* gene expression with several intrinsically chemotherapy-resistant cancers and increased expression of the *MDR1* gene in certain cancers with acquired drug resistance. These findings indirectly indicate that the *MDR1* gene contributes to multidrug resistance in many human malignancies [8]. However, direct evidence that the expression of this gene confers multidrug resistance *in vivo* is provided by studies on transgenic mice expressing the human *MDR1* gene, at levels comparable to those expressed in drug-resistant tumours [9–11].

A variety of agents are known for the ability to reverse *in vitro* the drug resistance phenotype of malignant cells. These agents are chemical inhibitors (chemosensitisers) of the multidrug transporter (P-glycoprotein) activity (for review, see [12]). These compounds, mostly lipophilic, amphipathic and heterocyclic substances, include calcium channel blockers, calmodulin antagonists, anthracyclines and vinca alkaloid derivatives, steroids and steroid analogues, cyclosporins and miscellaneous hydrophobic, cationic substances [12]. Unfortunately, most chemosensitisers currently in use in the clinic are also potent pharmacological agents with undesirable toxic effects at the therapeutic doses required to antagonise multidrug resistance. Therefore, new agents with fewer side-effects are urgently needed by oncologists [12].

The indole carbazole (K-252a) family of protein kinase inhibitors are very potent anti-proliferative agents that manifest cytotoxic activity against mammalian cells at a range of 0.1–200 nM [13]. Staurosporine, a member of this family, inhibits protein kinase C (PKC), as well as a variety of other kinases, with an exceptionally high potency [14]. It has been previously reported that staurosporine binds to P-glycoprotein and enhances drug accumulation in multidrug-resistant cells [15]. Several other studies have suggested that P-glycoprotein is phosphorylated, and that its activity might be modulated by several protein kinases, including PKC [16–20]. In view of these findings, and since staurosporine is very toxic to cells both *in vitro* and *in vivo* [21], several less toxic protein kinase modulators were tested here for their ability to sensitise multidrug-resistant cells to cytotoxic drugs. KT-5720, a derivative of K-252a, was found to be a potent new chemosensitiser. The chemosensitising activity of KT-5720 on the tested multidrug-resistant cells is apparently independent of its kinase inhibitory effects.

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MATERIALS AND METHODS

Drugs and chemosensitisers

Cytotoxic drugs were obtained from Sigma Chemicals (St Louis, Missouri, U.S.A.). Staurosporine, calphostin C, K-252a [(8R*, 9S*, 11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2, 3, 9, 10-tetrahydro-8, 11-epoxy-1H, 8H, 11H-2, 7b, 11a-triazadibenzo (a,g) cycloocta (c,d,e) trindene-1-one], K-252b (9-carboxylic acid derivative of K-252a), KT-5720 and KT-5822 (9-methoxy derivative of K-252a) were prepared at Kyowa Hakko Kogyo, Tokyo Research Laboratory (Tokyo, Japan). Phorbol-12-myristate-13-acetate (PMA) and 4α-phorbol-12-myristate-13-acetate (4α-PMA) were purchased from LC Services Co. (Wilburn, Massachusetts, U.S.A.). 2-[1-(3-dimethylaminopropyl)-indol-3yl]-3-(indol-3-yl)maleimide (GF-109203X), a synthetic derivative of staurosporine, was a generous gift of Dr H. Coste (Glaxo Laboratories, Les Ulis, France). Stock solutions of cytotoxic drugs and kinase modulators were prepared in dimethylsulphoxide (DMSO) at final concentrations of 10 mg/ml and 2 mM, respectively, and were stored at −20°C in the dark. Compounds were added directly from stock solutions to the culture media or were further diluted, keeping DMSO concentration below 0.1% (a non-toxic concentration in control experiments). Calphostin-treated cultures were pre-exposed to a neon light source (40 W, 50 cm) for 45–60 min to photoactivate this compound prior to the addition of colchicine and vinblastine [21]. All these compounds were tested *in vitro*, prior to our experiments, for their potency and efficiency in modulating protein kinase activities in a cell-free system, as described elsewhere [22].

Tissue culture and cell lines

T-25, T-25-Adh and HU-1 (T-25-Adh/Col^{R}-1000) cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% horse serum, penicillin and streptomycin. KB-3-1 (drug-sensitive carcinoma) and KB-V1 (vinblastine-resistant carcinoma) cells were cultured in the same medium supplemented with 10% fetal calf serum.

Transduction of variant S49 lymphoma cells with MDR1-*containing virus*

T-25-Adh cells [23] were plated at a density of 1×10^6 cells/dish and were transduced with ψ2-*MDR1*/A-2 virus, using a procedure described elsewhere [24]. Forty-eight hours postvirus inoculation, cells were initially selected for drug resistance in the presence of colchicine at 10 ng/ml. At this drug concentration, the number of colchicine-resistant colonies was approximately 1×10^4/dish, while control, non-transduced T-25-Adh cells were entirely sensitive, showing no drug-resistant colonies. Non-transduced T-25-Adh cells have demonstrated spontaneous resistance to colchicine only at a frequency lower than 1×10^{-8}. The transduced colchicine-resistant T-25-Adh cells (~10^4 drug-resistant pooled colonies) were further grown at increasing colchicine concentrations to select for cells greatly expressing the *MDR1* gene. The strategy used during this selection was a stepwise elevation of drug, so that no more than 50% of the inoculated cells died at each step. The rationale for using such a gradual selection strategy was to prevent selection of low frequency mutated cells that might arise under a high selection pressure. Using this selection procedure, we established a cell line termed HU-1 (T-25-Adh/Col$^{R\text{-}1000}$). This cell line is resistant to colchicine at 1 μg/ml and is approximately 200-fold more resistant to colchicine than its parental T-25-Adh cell line.

Preparation and analysis of genomic DNA

High molecular weight genomic DNA was isolated from T-25-Adh and HU-1 cells and analysed as described previously [9]. Briefly, DNA samples (20 μg each) were digested with *Eco*RI, electrophoresed on 1% agarose 1 × TBE buffer, and transferred to nitrocellulose paper. The 1.4-kb *Eco*RI *MDR1* probe was obtained from *pMDR5A*. Hybridisation was performed with nick-translated probe at 42°C in 50% formamide–5 × SSC (0.15 M NaCl plus 0.015 M sodium citrate)–2 × Denhardt solution (0.04% bovine serum albumin, 0.04% Ficoll, 0.04% polyvinylpyrrolidone)–20 mM sodium phosphate (pH 6.8)–10% dextran sulphate–100 μg of sonicated salmon sperm DNA per ml, followed by washing with 0.1 × SSC–1% sodium dodecyl sulphate (SDS) at 60°C.

Cytotoxicity assays

The sensitivity of cells to cytotoxic agents and/or protein kinase (PK) modulators was determined in a monolayer growth assay. For this assay, 5×10^4 cells in drug-containing medium were plated in each well of a 24-well tissue culture plate. Ninety-six hours later plates were washed with phosphate-buffered saline and stained with 0.5% (w/v) methylene blue in 50% ethanol. In this procedure, only surviving stained cells (dark area) remain adherent to the plastic dish. The density of stained cells was estimated in a semi-quantitive manner, comparing stain intensity at various plated cell densities. This assay was used for initial fast screening of PK modulators for cytotoxicities and chemosensitising effects. For quantitative measurements, 96 h after continuous treatment with cytotoxic drug and/or PK modulator, cells were washed with phosphate-buffered saline and resuspended by manual pipetting in 2 ml phosphate-buffered saline without calcium and magnesium. Samples were stained with trypan blue and the viable unstained cells were counted in a standard haemacytometer.

Chemosensitisation assay

This assay was performed in a 24-well tissue culture plate, as described above. Using these plates, each row of wells contained a fixed concentration of a single cytotoxic agent (colchicine, vinblastine or doxorubicin) and each column of wells contained a fixed concentration of a putative chemosensitiser (protein kinase modulator). The first row, in which the cytotoxic agent was absent, was used to measure growth inhibition (cytotoxicity) of the putative chemosensitiser. For each concentration of chemosensitiser, the relative resistance value was calculated. Relative resistance is defined as the ratios of the IC_{50} (50% inhibitory concentration on cell growth) values for the cytotoxic drug in the presence and absence of a given (fixed) concentration of chemosensitiser [12]. Relative resistance is 1.0 (maximum value) when no chemosensitiser is used and 0.005 (minimum value) when complete chemosensitisation is achieved.

RESULTS

Establishment and characterisation of multidrug-resistant lymphoma cells

We transduced S49 lymphoma T-25-Adh cells with *MDR1* virus (Ψ2-*MDR1*/A-2). This ecotropic virus contains the human *MDR1* cDNA under the control of a Harvey-MuSV LTR promoter [24]. The T-25-Adh cells, that were previously developed in our laboratory, are adhesive, non-tumorigenic, immunogenic cells derived from the suspension growing, malignant cell variants (T-25) of S49 mouse lymphoma [23]. Because these mouse lymphoma cells are adherent to tissue culture dishes, it is possible to develop simple plate assays of their survival. Since these cells are easily released from the plastic dish, cell counts are simple with no need for a standard trypsin/EDTA procedure, therefore, avoiding cell damage or aggregation. From these *MDR1*-transduced cells, a cell line, termed HU-1 (T-25-Adh/Col$^{R\text{-}1000}$), was established as described in Materials and Methods. HU-1 cells are resistant to colchicine at 1 μg/ml and are approximately 200-fold more resistant to colchicine than the parental T-25-Adh cells.

Southern blot hybridisation, using a human *MDR1* probe on genomic DNA isolated from HU-1 cells, revealed a unique 3.1-kb, *Eco*RI-digested fragment, as expected if the human *MDR1* cDNA is integrated into the T-25-Adh genome (Figure 1). HU-1 cells were similar to the parental T-25-Adh cells in terms of their rate of growth, adhesiveness (adherence to tissue culture dish), and Na^+/H^+ exchanger activity (data not shown). The last criterion indicates no diversity of another irrelevant pump system activity. Moreover, *in vivo* experiments, involving cell inoculation in mice, have indicated that HU-1 cells are also indistinguishable from the parental T-25-Adh cells by their ability to immunise syngeneic BALB/c mice against the tumorigenic T-25 cells [23].

Drug sensitivity studies on HU-1 cells have shown cross-resistance to colchicine, doxorubicin, vinblastine, and acti-

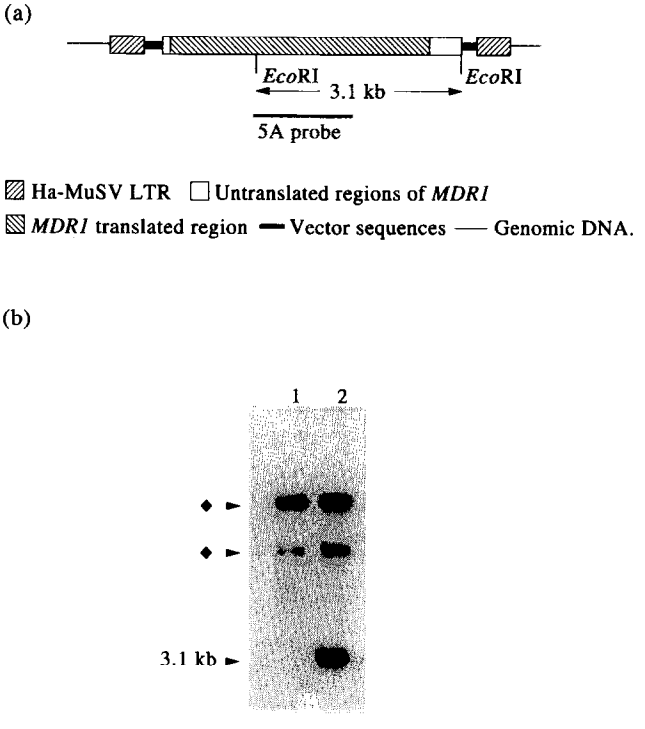


Figure 1. Southern hybridisation of genomic DNA from T-25-Adh cells transduced with Ψ2-*MDR1*/A2 virus. (a) Diagrammatic representation of the *MDR1* cDNA integrated into the mouse genome. After *Eco*RI digestion of a mouse genomic DNA and hybridisation with the 5A probe, derived from the central part of *MDR1* cDNA, a 3.1-kb labelled fragment is expected if the transgene is integrated. (b) Southern blot analysis of genomic DNA (20 μg) digested with *Eco*RI and hybridised with the 5A probe under conditions described in Materials and Methods. Genomic DNA isolated from: T-25-Adh (control) cells (lane 1); T-25-Adh *MDR1*-transduced cells that were selected with colchicine (lane 2). ◆, mouse endogenous *mdr* fragment; 3.1 kb, human *MDR1* expected fragment.

Table 1. Characterisation of cross-resistance in HU-1 and KB-V1 cells

Cell line	Relative resistance to cytotoxic drugs COL	VBL	DOX	ACT
Lymphoma cell lines				
T-25-Adh	1	1	1	1
HU-1 (T-25 Adh/Col$^{R\text{-}1000}$)	210	90	36	27
KB cell lines*				
KB-3-1	1	1	1	NM
KB-V1	170	210	420	NM

For cross-resistance tests, cell were plated at 5×10^2 per 35-mm dish. Drugs were added 16 h after cell inoculation. Drug-resistant colonies were counted after 10 days of incubation at 37°C. Relative resistance to drugs (COL, colchicine; VBL, vinblastine; DOX, doxorubicin; ACT, actinomycin D) was calculated by dividing the LD_{50} value for each cell line (the concentration reducing cloning efficiency by 50%) by the LD_{50} value for the parental drug-sensitive cell lines.

* These results were previously published [25], and are shown for comparison purposes only. Relative resistance determined using the same colony-forming assay as described above. NM, not measured. The values in the table are means of duplicate experiments, S.D. ± 2.5%.

nomycin D (Table 1), similarly to cross-resistance described for other cell lines that were selected with a single drug from the MDR1 family [3, 7]. It should be noted, however, that the cross-resistance pattern of these HU-1 cells to multiple drugs is somewhat different from the cross-resistance pattern of KB-V1 cells, a human cell line that overexpresses P-glycoprotein [25]. The reason for this is that the *MDR1* retrovirus used for transduction encodes a mutant transporter with a gly 185 → val 185 mutation that enhances resistance to colchicine compared to vinblastine [26]. Verapamil, a calcium channel blocker, is known for its ability to reverse multidrug resistance by competitively inhibiting drug transport out of drug-resistant cells [12, 27]. As expected, verapamil reversed the colchicine resistance (IC_{50} = 0.9 μM) of HU-1 cells (Figure 2). Taken together, these data indicate that HU-1 cells can be used in studies involving the reversal of multidrug resistance. Moreover, as shown in Figure 2a, the adhesive properties of this lymphoma-derived cell line allow a simple and rapid (72–96 h) assay for the screening of new chemosensitisers.

Chemosensitisation and toxicity screening of protein kinase modulators

Several studies have shown that P-glycoprotein is phosphorylated and that its activity might be modulated by cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC) and protein kinase P [16–20]. Moreover, it has been reported that staurosporine, a potent PKC and PKA inhibitor, binds to P-glycoprotein and enhances accumulation of vincristine in

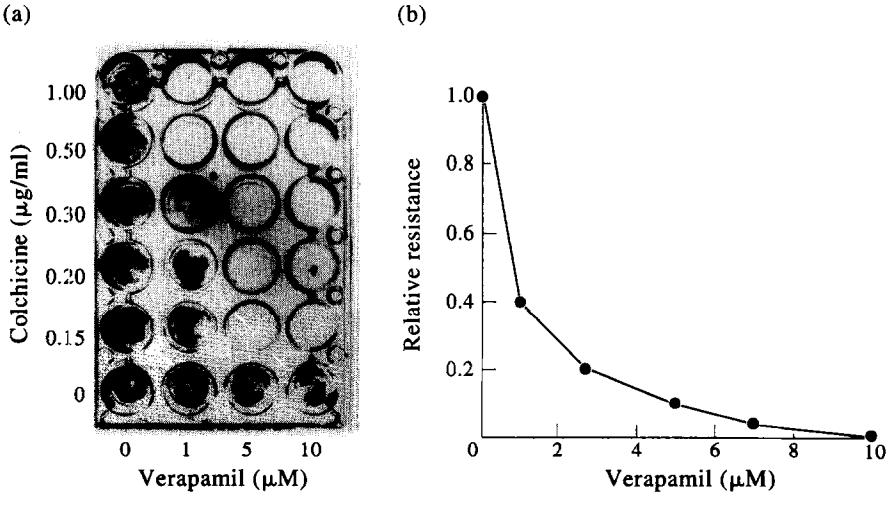
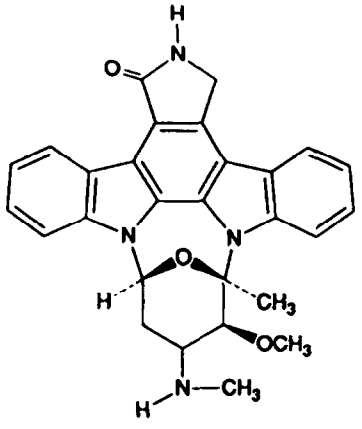


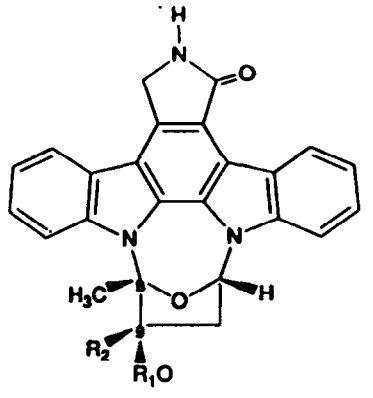
Figure 2. Chemosensitisation of HU-1 cells by verapamil. (a) A monolayer growth assay in a 24-well tissue culture plate, as described in Materials and Methods. Each row of wells represents a fixed concentration of colchicine; each column of wells represents a fixed concentration of verapamil. (b) Graphic representation of drug-resistance reversing pattern by verapamil. Relative resistance (RR) is determined according to the formula: RR = IC_{50} (colchicine + verapamil)/IC_{50} (colchicine). IC_{50} is 50% inhibitory concentration on cell growth. Relative resistance is 1.0 (maximum value) when no chemosensitiser is used, and 0.005 (minimum value) when complete chemosensitisation is achieved. The data are means from duplicate experiments, S.D. ± 5%.

multidrug-resistant cells [15]. Therefore, we tested the effect of known protein kinase modulators on our multidrug-resistant HU-1 cells. The chemical structures of these modulators are shown in Figure 3. All these substances were pretested *in vitro* for their potency and efficiency to modulate protein kinase activities (Table 2). The cytotoxicities of these compounds on HU-1 cells are depicted in Figure 4. The cytotoxicity values(IC_{50}) of these compounds in the multidrug-resistant HU-1 cells were identical to the values found for the parental, drug-sensitive T-25-Adh cells.

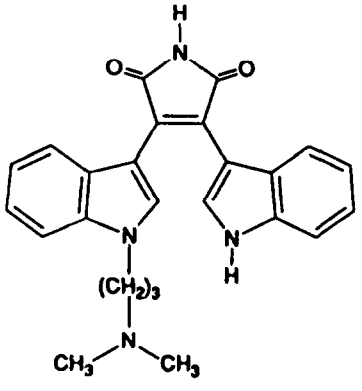
To test whether the above-mentioned protein kinase modulators can reverse drug resistance in HU-1 cells with 96-h treatment, we incubated these cells with increasing concentrations of various protein kinase modulators, each in the absence or the presence of various fixed concentrations of colchicine or vinblastine (Table 3). These compounds were tested in this assay from non-toxic to sub-toxic concentrations (as indicated in Figure 4) up to concentrations that inhibited 90% cell growth in the absence of cytotoxic agents (Table 3). The chemosensitising effects of these compounds in the chemosensitisation assay, expressed as sensitisation ratios which are LD_{50} values to cytotoxic agents in the absence and the presence of a PK modulator (Table 3), are depicted in Table 3. These results indicate that neither the protein kinase C activator (PMA) nor protein kinase C inhibitors (staurosporine, calphostin C, GF109203X, K-252a and K-252b) could chemosensitise HU-1 cells at sub-toxic concentrations. KT-5822 and K-252a, both inhibitors with relative selectivity towards cGMP-dependent protein kinase activity, did not show any chemosensitising properties on HU-1 cells. However, the compound KT-5720 was found to be a very potent chemosensitiser for HU-1 cells with sensitisation ratios of 193 and 97 for colchicine and vinblastine, respectively, in the presence of 10 μM of KT-5720 (Table 3). The effect of colchicine on HU-1 cells in the presence of KT-5720, as well as the effect of colchicine alone on the parental T-25-Adh drug-sensitive cells, could be detected under the microscope as early



Staurosporine



K252aDerivatives



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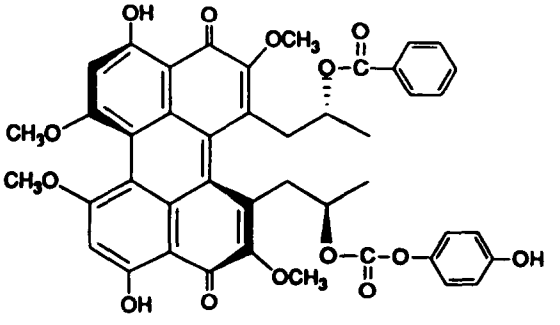

Calphostin C

Figure 3. Chemical structure of K-252a derivatives and other protein kinase inhibitors. Structures of the side chains of K-252a are: R1 = H; R2 = $COOCH_3$. As indicated in Table 2, derivatives of K-252a are either chemically modified at R1 or R2 side chains.

Table 2. In vitro *inhibitory effects of K-252a-related compounds on major cellular protein serine/threonine kinases**

Compound	Side chain composition R1	Side chain composition R2	Protein kinases *In vitro* inhibitory constants, *Ki* (nM) PKA	PKG	PKC	MLCK	CaMK
K-252a	H	$COOCH_3$	10–20	15–20	25	20	2
K-252b	H	COOH	90	100	20	150	12
KT-5720	H	$COO(CH_2)_5CH_3$	60	>2000	>2000	>2000	n.t.
KT-5822	CH_3	$COOCH_3$	>40	2–4	80	>10000	n.t.
Staurosporine			7–20	8–10	0.7–10	1.3	10–100
Calphostin C			900–70000	n.t.	4–100	n.t.	>10000

* Inhibitory activities presented as *Ki* (nM). PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; MLCK, myosin light chain kinase; CaMK, calcium calmodulin type 2 kinase; n.t., not tested.

as after 15 h of incubation. At this stage, the morphology of the drug-sensitive cells was dramatically changed, and cells became at least 3-fold larger in diameter with no distinct nuclear structure.

Although KT-5720 is also a potent inhibitor with relative selectivity towards cAMP-dependent protein kinase (PKA) activity [22], it seems that the chemosensitising properties of this compound are independent of its protein kinase inhibitory effects. This is so, since the other PKA inhibitors, such as K-252a, KT-5822 and staurosporine, could not reverse colchicine and vinblastine cross-resistance in HU-1 cells. Moreover, since K-252a, KT-5822 and staurosporine are more toxic (IC_{50} values of 30, 500 and 0.6 nM, respectively) than KT-5720 (IC_{50} value of 10.5 μM) to HU-1 cells, it does not seem that a diversity in membrane permeability towards the non-chemosensitising inhibitors could explain the fact that KT-5720 can solely reverse colchicine and vinblastine resistance in HU-1 cells. It should be noted that at the concentration required to completely reverse colchicine or vinblastine resistance (both approximately at 10 μM), KT-5720 reduced cell density to 60%. However, at this concentration, KT-5720 did not have any cell-killing effect, since no dead cells (trypan blue stained cells) could be detected at 10 μM KT-5720 in the absence of colchicine or vinblastine. At this concentration, only a decrease in cell division rate was observed. This moderate anti-proliferative effect of KT-5720 might even add to its chemosensitising property as a potent drug in future combination chemotherapy.

Chemosensitising properties of KT-5720 on multidrug-resistant cells

Since KT-5720 was initially found as a potent colchicine-sensitiser on HU-1 cells, we tested its potency to reverse cross-resistance towards other cytotoxic drugs that are also excluded by P-glycoprotein in multidrug-resistant cells. The chemosensitising effects of KT-5720 on colchicine (primary resistance) and on doxorobicin or vinblastine (cross-resistance) were compared (Figure 5a). These results demonstrate that the multiple drug resistance caused by these three cytotoxic drugs can be fully reversed by KT-5720, at a concentration of 10 μM. However, it should be noted that, in HU-1 cells, the 50% inhibitory dose (IC_{50}) of KT-5720 on vinblastine and doxorubicin resistance (IC_{50} values 1.2 and 1.5 μM, respectively) is at least 2-fold lower than the IC_{50} value (3.0 μM) for colchicine resistance. This phenomenon, where a chemosensitiser appears to have greater effect on cross-resistance than on primary resistance to selecting agents, has been previously reported for verapamil [12, 25].

To test whether KT-5720 could sensitise other commonly used multidrug-resistant cells, we examined the effect of KT-5720 on human carcinoma KB-V1 cells [25], which are resistant to vinblastine. These experiments demonstrated that KT-5720 could reverse vinblastine resistance (IC_{50} value 0.5 μM) of KB-V1 cells (Figure 5b). However, this chemosensitiser appears to have a 6-fold greater effect on the primary resistance to the selecting agent in KB-V1 cells than in HU-1 cells (IC_{50} values 0.5 and 3.0 μM, respectively). This difference might be explained by different post-translational modifications in P-glycoprotein of murine versus human cells. As previously suggested, such modifications might cause changes in the affinity of cytotoxic drugs or KT-5720 for the putative drug binding site(s) [12]. It is also possible that the variation in the chemosensitisation pattern between KB-V1 and HU-1 cells might be explained by the alternate single amino acid change (glycine → valine, respectively) at position 185 of P-glycoprotein. This single amino acid alternation has been previously reported to be responsible for an altered pattern of multidrug resistance [26], and recent results suggest that this mutation may also change patterns of inhibition of P-glycoprotein (C. Cardarelli, W. Stein, I. Pastan and M.M. Gottesman, unpublished data). Although the chemosensitising properties of KT-5720 have not been evaluated in other multidrug-resistant cell lines, our results, showing that KT-5720 can reverse drug resistance in two different cell lines (mouse lymphoma and human carcinoma), suggest that KT-5720 might act as a general chemosensitiser.

We also tested the effect of K-252a, a potent inhibitor of the protein kinases PKA, PKC and PKG, on resistance to vinblastine (primary resistance) and colchicine (cross-resistance) in KB-V1 cells. Similarly to HU-1 (Table 3), KB-V1 cells could not be sensitised to cytotoxic drugs by K-252a at sub-toxic concentrations (1–100 nM). The IC_{50} values of K-252a, from three independent experiments, in the absence of cytotoxic agents and in the presence of vinblastine (1 μg/ml) or colchicine (0.6 μg/ml) are 36 ± 2, 37 ± 3 and 34 ± 2.5 nM, respectively. Therefore, although the mechanism of chemosensitisation by KT-5720 is still unclear, our data suggest that the chemosensitisation property of KT-5720 is independent of its protein kinase inhibitory effects.

DISCUSSION

To overcome multidrug resistance in cancers, one major strategy already undergoing clinical trials is the reversal of the drug resistance phenotype of tumours, using chemical inhibitors

Table 3. The relationship between protein kinases' specificity, cytotoxicity and chemosensitising properties of K-252a-related compounds

Compound	*In vitro* relative protein kinase specificity*	Cytotoxic agent (μg/ml)† IC_{50} for cell growth (μM) None	COL^{1}	$COL^{0.5}$	$COL^{0.2}$	$VBL^{0.4}$	Concentrations tested for sensitisation‡	LD_{50} values (μg/ml) for cytotoxic agents within tested range§ COL	VBL	Sensitisation ratio for cytotoxic agents within tested range** COL	VBL
K-252a	PKC=PKA=PKG	0.030	0.028	0.031	0.030	0.033	1–100 nM	1.1	0.45	1	1
K-252b	PKC>PKA>PKG	0.42	0.45	0.040	0.043	0.39	0.01–10 μM	1.1	0.45	1	1
KT-5720	PKA>>PKC>>PKG	10.5	0.67	1.35	5.40	0.33	0.1–12 μM	1.1–0.0057‖	1–0.0046¶	1–193	1–97
KT-5822	PKG>PKA>PKC	0.51	0.53	0.48	0.51	0.52	0.01–0.8 μM	1.1	0.45	1	1
GF109203X	PKC>>PKA	20.0	19.8	20.1	20.0	n.t.	0.1–30 μM	1.1	n.t.	1	n.t.
Calphostin C	PKC>>PKG>PKA	0.10	0.10	0.09	0.10	0.11	1–200 nM	1.1	0.45	1	1
Staurosporine	PKC>>PKA>PKG	0.0006	0.0006	0.0007	0.0006	0.0007	0.01–2 nM	1.1	0.45	1	1
PMA	PKC	0.005	0.005	0.006	0.005	0.005	0.5–20 nM	1.1	0.45	1	1
4a-PMA	PKC	10.0	10.3	9.9	10.2	9.7	0.1–30 μM	1.1	0.45	1	1

* Summary of protein kinase specificity according to Table 2. PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase. † The IC_{50} of these compounds on HU-1 cells were measured in a 96-h monolayer growth assay, as described in Materials and Methods, in the absence or the presence of various fixed concentrations of cytotoxic agents. COL^{1}, colchicine at 1 μg/ml; $COL^{0.5}$, colchicine at 0.5 μg/ml; $COL^{0.2}$, colchicine at 0.2 μg/ml; $VBL^{0.4}$, vinblastine at 0.4 μg/ml. ‡ The compounds were tested for chemosensitising ability in the indicated range up to concentrations that inhibited 90% cell growth in the absence of cytotoxic agents, as described in Figure 4. § The LD_{50} values for a cytotoxic agent (colchicine or vinblastine) were tested in the absence or presence of the PK modulator at several fixed concentrations within the indicated range. ‖ LD_{50} values for colchicine at 0, 0.1, 1, 5 and 10 μM of KT-5720 are 1.1, 1.1, 0.88, 0.62 and 0.0057 μg/ml, respectively. ¶ LD_{50} values for vinblastine at 0, 0.1, 1, 5 and 10 μM of KT-5720 are 0.45, 0.45, 0.26, 0.11 and 0.0046 μg/ml, respectively. ** Sensitisation ratio (SR) is determined according to formula: SR = LD_{50} (cytotoxic agent)/LD_{50} (cytotoxic agent + PK modulator). Cytotoxic agent is either colchicine or vinblastine. The values in the table are means of three independent experiments, S.D. ± 4.5%. n.t., not tested.

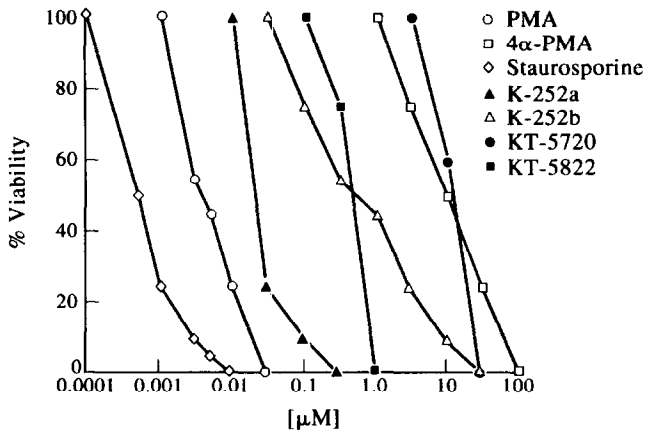

Figure 4. Cytotoxicity dose-response curves of protein kinase modulators (K-252a derivatives and phorbol esters) on HU-1 cells. Cytotoxicity is measured as % of cell viability as calculated from cell densities in the presence and absence of a given compound. Values are means of triplicate experiments, S.D. ± 5.1%.

(chemosensitisers) of the multidrug transporter (P-glycoprotein). As part of research to develop new, less toxic, natural and synthetic compounds to inhibit the multidrug transporter, we developed a new assay for P-glycoprotein inhibitors based on *MDR1* retrovirus-transduced adherent S49 lymphoma cells and have used this system to test various protein kinase inhibitors. It has been previously reported that P-glycoprotein is phosphorylated and regulated by PKA and PKC inhibitors [15–20]. However, whether these inhibitors reverse the multidrug resistance of cells through the involvement of their kinase inhibition on the function of P-glycoprotein remains unclear. In this study, we did not find any correlation between the known inhibitory effect of K-252a derivatives on major cellular protein kinases and their apparent ability to reverse multidrug resistance. This conclusion is also supported by recent findings indicating that staurosporine derivatives inhibited multidrug resistance, regardless of their protein kinase inhibitory effects [28, 29]. It has also been reported [30] that KT-5720 inhibits a 170-kDa membrane-associated protein kinase activity that is present in some, but not all, multidrug-resistant cell lines, including KB-V1. Yet, P-glycoprotein is not a substrate of this protein kinase [30]. Therefore, although the mechanism of drug resistance inhibition by KT-5720 is still unknown, our data add to the growing evidence [28–30] that phosphorylation of P-glycoprotein may not be a universal mechanism for modulation of drug resistance, as has been previously suggested.

In our study, the compound KT-5720 had a potent chemosensitising effect on multidrug resistance of the highly drug-resistant HU-1 and KB-V1 cells. The potency and selectivity of KT-5720, when compared to its closely related analogues (K-252a, K-252b and KT-5822), emphasises the importance of the aliphatic side chain on R2 (Figure 3 and Tables 2 and 3).

The fungal alkaloid K-252a family of protein kinase inhibitors is also known to contain very potent anti-proliferative agents [14]. Staurosporine, a member of this family, is also an effective anti-tumour agent *in vivo*, according to studies of human bladder carcinoma [21]. The possible importance of staurosporine and other K-252a derivatives, as putative new anti-cancer drugs, is suggested by their ability to antagonise tumour cell invasion [31]. A recent study on the K-252a derivatives, including KT-5720, has demonstrated anti-proliferative and differentiation effects on pheochromocytoma cells that are independent

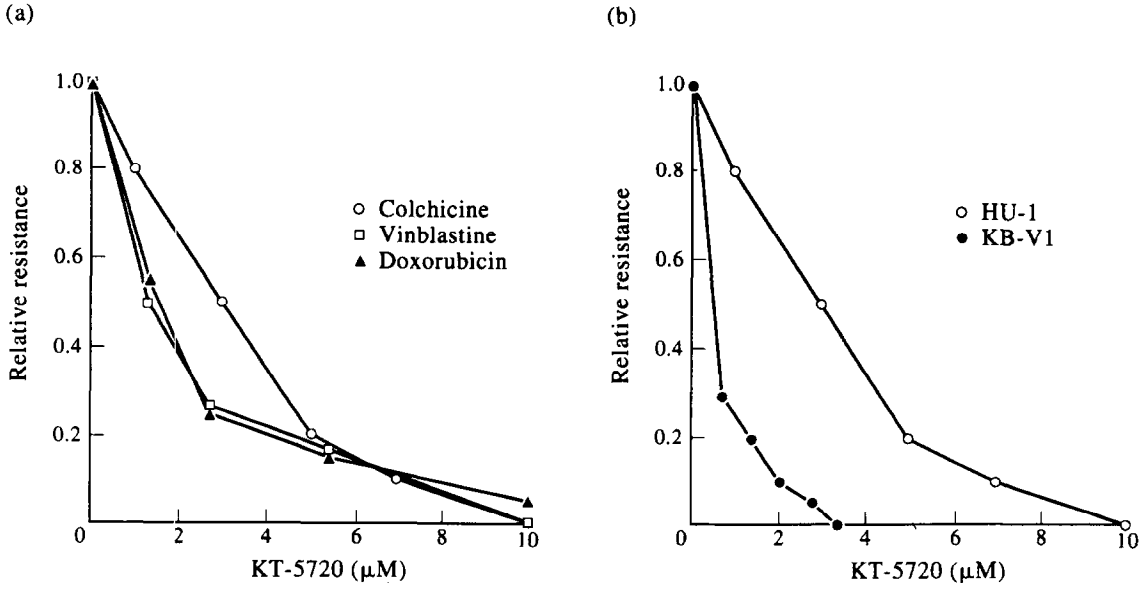

Figure 5. Reversal of drug resistance by KT-5720. Relative resistance is defined in Materials and Methods. (a) Reversal of multiple drug resistance by KT-5720 in HU-1 cells. (b) Reversal of primary resistance by KT-5720 in HU-1 and KB-V1 cells. The effect of KT-5720 on: ○, colchicine resistance in HU-1 cells; ●, vinblastine resistance in KB-V1 cells. The data represent means of triplicate experiments. S.D. values were ±4.8%, ±3.6% and ±4.1% for colchicine, vinblastine and doxorubicin, respectively in (a). In (b), S.D. values were ±4.8% and ±4.2% for HU-1 and KB-V1, respectively.

of PKC inhibition [32]. Since KT-5720 fully reversed multidrug resistance in our cell lines at non-toxic concentrations, it might be a candidate as a new clinical chemosensitiser in combination chemotherapy. Moreover, its suggested anti-proliferative and anti-invasiveness effects [31, 32] might even increase the potency of this compound in future cancer therapy.

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