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Original Paper

Tunicamycin Potentiates Drug Cytotoxicity and Vincristine Retention in Multidrug Resistant Cell Lines

D. Hiss,[1] G. Gabriels,[1] P. Jacobs[2,*] and P. Folb[1]

[1]Department of Pharmacology, University of Cape Town, Medical School; and [2]University of Cape Town Leukaemia Centre and Department of Haematology, Groote Schuur Hospital, Observatory 7925, Cape Town, South Africa

Tunicamycin (TM), an inhibitor of glycoprotein processing, was investigated for its potential to reverse the multiple drug resistance (MDR) phenotype. When TM was added *in vitro* to drug-resistant NIH-3T3-MDR and KB-8-5-11 cells, they developed an increased sensitivity to doxorubicin, epirubicin, vincristine and colchicine. Similarly, the sensitivity of NIH-3T3-MDR cells to cisplatin was also enhanced by TM. In the presence of TM, drug-sensitive NIH-3T3-parental cells exhibited greater susceptibility to the toxic effects of doxorubicin, epirubicin, vincristine (marginally significant), and colchicine, but not to cisplatin. Tunicamycin-treated drug-sensitive KB-3-1 cells showed an increased response to vincristine, but not to the other anticancer drugs. Pretreatment with TM inhibited glycoprotein synthesis in all the cell lines. Neither prior exposure to, nor co-incubation with TM, influenced the uptake of vincristine (VCR) in the various cell lines. However, NIH-3T3-MDR cells accumulated less VCR than their drug-sensitive controls and also exhibited reduced efflux of the drug when treated with TM. There were no significant differences in the levels of intracellular VCR uptake between drug-sensitive KB-3-1 and KB-8-5-11 cells. Tunicamycin increased intracellular VCR retention in KB-8-5-11 and NIH-3T3-MDR cells, but not in NIH-3T3-parental cells. However, drug-sensitive KB-3-1 cells expressed reduced VCR retention in response to TM exposure, indicating that correlations between VCR toxicity and its retention in the presence of TM should be made with caution. The results suggest that the enhancement of intracellular VCR retention in MDR cell lines caused by TM is likely to be the result of inhibition of VCR efflux. Inhibition of glycoprotein synthesis during TM exposure may account for the changes in VCR efflux and retention observed in the MDR cell lines. The enhancement of cisplatin cytotoxicity in NIH-3T3-MDR cells after exposure to TM is an interesting observation, since it is generally believed that agents which modify the MDR phenotype do not show a sensitising effect to cisplatin. These findings may have applications in the reversal of drug resistance.

Key words: tunicamycin, cytotoxicity, anticancer drugs, modulation of drug resistance, resistance modifiers, glycoproteins, vincristine transport

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INTRODUCTION

INTRINSIC OR acquired resistance of tumour cells to antineoplastic drugs is the primary cause of chemotherapeutic failure. Advances in cancer research have refined our understanding of clinical and experimental drug-resistance mechanisms, and have raised expectations that the rational design of strategies to circumvent the problem may have potential clinical applications [1, 2]. Cells exposed to a single (selecting) cytotoxic agent frequently develop cross-resistance to several pharmacological classes of structurally and functionally dissimilar drugs, a phenomenon termed

Correspondence to D. Hiss.
*Present address: Department of Haematology, The Wynberg Hospital, P.O. Box 18171, Wynberg 7824, South Africa.
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multidrug resistance (MDR). Multidrug resistance is often associated with the overexpression of a plasma membrane phosphoglycoprotein. P-glycoprotein, a product of the *MDR*1 gene, protects cells against a broad spectrum of antitumour compounds by functioning as an energy-dependent drug efflux pump [3–6]. Thus, the extent of penetration into and accumulation and retention of anticancer drugs within tumour cells is an important determinant of their cytotoxicity [7–9]. Multidrug-resistant cells accumulate less anticancer drug than their parental counterparts [10, 11], attributable to active efflux mediated by P-glycoprotein.

The plasma membrane has long been regarded as a site for chemotherapeutic intervention [7]. Presumably, P-glycoprotein is strategically positioned at the cell surface to mediate a complex array of interactions between the cell and its external and internal environments [12, 13]. P-glycoprotein is modified post-translationally by N-glycosylation [14], which probably regulates its activity. It is possible to interfere with the biosynthesis of N-glycans, the biological consequences of which include enhanced susceptibility of proteins to proteases, improper protein processing and misfolding of polypeptide chains, loss of biological activity [15] and sensitisation of cells to cancerostatic protein toxins [16]. Glycoprotein processing inhibitors may, therefore, prove valuable in enhancing the actions of anticancer agents. Modification of P-glycoprotein can also be applied usefully to overcome MDR. Chemosensitisers represent an important advance to this goal [2, 17].

In this study, we have shown that supression of glycoprotein synthesis with tunicamycin, a potent inhibitor of protein N-glycosylation [18], enhances the sensitivity of multidrug-resistant KB carcinoma cells and NIH-3T3 cells transfected with the *MDR*1 gene to cytotoxic agents, and increases their retention of radiolabelled vincristine (VCR).

MATERIALS AND METHODS

Drugs and chemicals

Doxorubicin and epirubicin (Farmitalia Carlo Erba, Milan, Italy), cisplatin (Lennon, South Africa), vincristine sulphate, colchicine and MTT 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), trypsin 1:250 (Difco Laboratories, Detroit, Michigan, U.S.A.), phosphate-buffered saline (PBS Dulbecco 'A', Oxoid, U.K.), tissue cultue media, penicillin G and streptomycin sulphate (Gibco, U.K.), tuni- camycin™ (Boehringer Mannheim, Germany), EDTA and DMSO (Merck Chemicals, Germany), gentamicin sulphate (cidomycin®, Roussel Laboratories, South Africa), radioactive isotopes (Amersham, U.K.) and Bio-Rad™ protein assay dye reagent concentrate (Bio-Rad Laboratories Ltd, U.K.) were used in this study. All other reagents were of analytical grade and were obtained from either Merck Chemicals or Sigma Chemical Co.

Cell lines and culture conditions

The human epidermoid carcinoma cell line, KB-3-1, and its MDR derivative, KB-8-5-11, which contains the amplified *MDR*1 gene encoding P-glycoprotein [19], were provided by M.M. Gottesman (National Cancer Institute, Bethesda, Maryland, U.S.A.). The isolation and characteristics of the KB carcinoma cell lines have been previously described [10, 20]. The NIH-3T3 parental murine fibroblasts and NIH-3T3-MDR cells, derived by transfection of the NIH-3T3 parental cells, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (HIFCS), penicillin G (100 U/ml) and streptomycin sulphate (100 μg/ml) or gentamicin sulphate (50 μg/ml); KB-8-5-11 and NIH-3T3-MDR cells, were maintained in DMEM as for parental cells with addition of 1 μg/ml colchicine. Incubator settings were calibrated to 37°C, 5% CO_2:air and 85% relative humidity. Cells were subcultured routinely with trypsin-EDTA (0.25–0.02%, w/v) in Ca^{2+}- and Mg^{2+}-free PBS and maintained in the logarithmic phase of growth. Cell lines were tested regularly and found to be free of mycoplasma contamination.

Precursor-incorporation-inhibition studies

The effects of prior exposure to TM on the incorporation of labelled precursor [^{3}H]mannose (30–60 Ci/mmol) into trichloroacetic acid-insoluble macromolecules (glycoproteins) were evaluated in the various cell lines. Following an initial 16 h exposure to TM, cells in the logarithmic phase of growth (usually 4×10^5 cells/ml/well) in 24-well plates were incubated in the absence or presence of TM (concentrations from 0.005 to 5 μg/ml), and 5–10 μCi/ml of [^{3}H]mannose in culture medium for time intervals of 4–36 h. After each incubation period, medium containing radioactive precursor was aspirated and the cells washed three times in ice-cold PBS and solubilised with 0.5 ml of 1% SDS/0.3 M NaOH. An aliquot (0.1 ml) was removed for protein determination. To another aliquot (0.4 ml) was added 1 ml of ice-cold 10% trichloroacetic acid (TCA), and the mixture was left on ice for 20–30 min. TCA-insoluble material was collected on to glass-fibre filters (diameter 25 mm, Schleicher & Schüll). Filters were washed twice with 2 ml ice-cold 10% TCA, twice with 3 ml 96% ethanol and once with chloroform:methanol:water (10:10:3, v/v) to remove glycolipids. Filters dried in air were placed in 20-ml scintillation vials to which 10 ml scintillation fluid (Beckman Ready-Protein™) were added for radioactive counting. Counting efficiencies were 33% for ^{3}H and 66% for ^{14}C. All samples were corrected for background radioactivity. Synthesis was standardised and expressed as the amount of DPM incorporated into trichloroacetic acid-insoluble fractions per total cell protein content.

Pretreatment of cells with tunicamycin

TM stock solutions were prepared by dissolving the contents of a 10-mg vial in 25 mM NaOH and diluting to 0.8 mg/ml tunicamycin and 10 mM NaOH with pyrogen-free distilled-deionised water. The solution was sterilised by passing through a 0.22-μm disposable filter (Millipore, Millex-GV) and stored at −20°C for a period not exceeding 3 weeks. Immediately before each experiment, the tunicamycin solution was diluted to a final concentration of 5 μg/ml in culture medium. This concentration was chosen because it had little effect on cell viability, which remained greater than 95% between 4 and 72 h of exposure, as assessed by trypan blue dye exclusion. Tunicamycin was tested for its potential to increase the cytotoxicity of doxorubicin (DOX), epirubicin (EPX), vincristine (VCR), colchicine (COL) and cisplatin (CPL). Cells were pretreated with tunicamycin for 16 h in order to inhibit glycoprotein synthesis prior to their exposure to the different drugs. Control cultures were

exposed to an equal volume of 10 mM NaOH (vehicle), the final concentration (62.5 μM) of which was confirmed not to affect the pH of the culture medium.

In vitro *cytotoxicity assays*

To determine the effect of tunicamycin on the cytotoxicity of DOX, EPX, COL, VCR and CPL, preconfluent cells from stock cultures were detached with trypsin-EDTA (0.25–0.02%, w/v) in PBS, washed twice with PBS and resuspended in complete culture medium to obtain single-cell suspensions. Cells were counted in an electronic particle counter (Coulter, Hialeah, Florida, U.S.A.). Standardisation of cell numbers in individual wells of a 96-well microtitre plate was confirmed by a linear correlation ($r = 0.97$) between cell number and absorbance up to a maximum density of $3.5–4.5 \times 10^4$ cells/well. Cells were seeded at a density of 3×10^3 cells/well in a final volume of 200 μl as follows: after trypsinisation, cells were washed twice in PBS, resuspended in 10 ml complete culture medium and repeatedly pipetted to ensure an homogeneous mixture during dispensing of 100-μl aliquot/well. The cells were then allowed to attach and grow for 48–72 h.

Cytotoxic drugs were dissolved in PBS and filter (0.22-μm) sterilised. The drugs were diluted in culture medium, free of phenol red, to avoid interference with spectrophotometric assays [22]. After the addition of drugs in various concentrations to wells, repeated eight times, cells were incubated in the presence or absence of tunicamycin for a futher 72 h. The cytotoxocity of drug in the tunicamycin-treated and tunicamycin-free cultures was determined by the MTT assay [22]. MTT (20 μl of 5 mg/ml in sterile PBS) was added to each well and the plates were incubated for 5 h at 37°C. Plates were then centrifuged at 400*g* for 5 min to pellet any floating cell aggregates. The supernatant was aspirated and the formazan crystals dissolved in DMSO (200 μl/well). The plates were then gently agitated for 10 min and the absorbance read at a sample wavelength of 540 nm and a reference wavelength of 630 nm on a microplate reader. The IC_{50} of the dose–response curve was defined as the drug concentration required to reduce the final absorbance to 50% of the control value. Dose–response curves were corrected for the decrease in absorbance caused by tunicamycin *per se*, which was always less than 10% of the control value. The sensitisation (potency) ratio and associated 95% confidence interval for each drug in the presence of tunicamycin was calculated by Fieller's ratio of means test as modified by Bliss [23].

Assay of vincristine uptake and efflux

To assay vincristine (VCR) uptake and efflux, cells were seeded at a density of 5×10^4 cells/ml in 24-well plates and allowed to grow for 48 h under standard conditions. Cells were pretreated with 5 μg/ml TM as described above. Parallel controls were set up. Total cellular accumulation of VCR was determined by exposing cells in quadruplicate wells to [G-^{3}H]VCR sulphate (30 nM) in the continued absence or presence of TM in a final volume of 0.5 ml for various incubation times. At the end of each incubation period, cells were washed three times with 1 ml ice-cold PBS and solubilised in 0.5 ml of 1% sodium dodecyl sulphate (SDS)/0.3 M NaOH. One aliquot (0.4 ml) was neutralised by the addition of 0.2 ml of 2 M acetic acid, mixed with 10 ml Beckman Ready-Solv EP^{TM} scintillation fluid and counted in a Beckman scintillation spectrometer. Intracellular drug at each time point was determined by subtracting the value for non-specific/surface-bound drug obtained by incubation with 100 μM unlabelled VCR for 10 sec at 0–4°C from the value for total drug. The other aliquot (0.1 ml) was assayed for total cellular protein. Vincristine efflux was determined by loading control and TM-pretreated cells with [^{3}H]VCR for 60 min (0-time value for efflux) followed by washing preloaded cells three times with ice-cold PBS and subsequently incubating at 37°C in serum- and antibiotic-free medium (2 ml) for various time intervals. The absence or presence of TM was maintained throughout the post-incubation periods. Cells were harvested as described for uptake studies.

Protein estimation

Total cellular protein was determined by the automated Bio-Rad^{TM} dye-binding microassay [24], using bovine serum albumin as standard.

Data analysis

IC_{50} values of cytotoxic drugs were estimated by the PHARM/PCS computer program [23]. Data given are means ± SEM of eight determinations from one representative experiment out of three, conducted independently, all of which yielded comparable results. Statistical analysis was performed on the variables in this study using the Student's two-tailed *t*-test. The level of significance was set at $P \leqslant 0.05$.

RESULTS

Effects of tunicamycin on glycoprotein synthesis

The incorporation of [^{3}H]mannose into glycoproteins in drug-resistant (NIH-3T3-MDR and KB-8-5-11) and corresponding drug-sensitive (NIH-3T3-parental and KB-3-1) cell lines in the absence (control) or presence of TM (TM-treated) was measured at different time intervals following an initial 16-h pre-incubation with the antibiotic. The level of radiolabelled mannose associated with trichloroacetic acid-insoluble material was significantly higher in KB-8-5-11 drug-resistant cells compared with KB-3-1 cells (Figure 1). At all incubation times studied, exposure of both cell lines to TM resulted in a decrease in the quantity of cell-associated [^{3}H]mannose-labelled macromolecules (Figure 1a). Similarly, the incorporation of [^{3}H]mannose by both NIH-3T3-parental and NIH-3T3-MDR was significantly inhibited by TM at the time intervals indicated (Figure 1b). Drug-resistant KB-8-5-11 and NIH-3T3-MDR cells showed a consistently greater rate of glycoprotein synthesis than their drug sensitive counterparts.

Potentiation of drug cytotoxicity by tunicamycin

The effects of TM on the viability of various drug-sensitive and drug-resistant cell lines following 72 h continuous exposure to the antibiotic were compared (data not shown). In all the cell types, TM did not affect cell viability in the concentration range 0.001–10 μg/ml and survival was consistently greater than 90% or similar to control (i.e. cells not treated with TM). Higher concentrations of the antibiotic (30–100 μg/ml) were necessary to achieve greater than 20% reduction in cell survival as measured by the

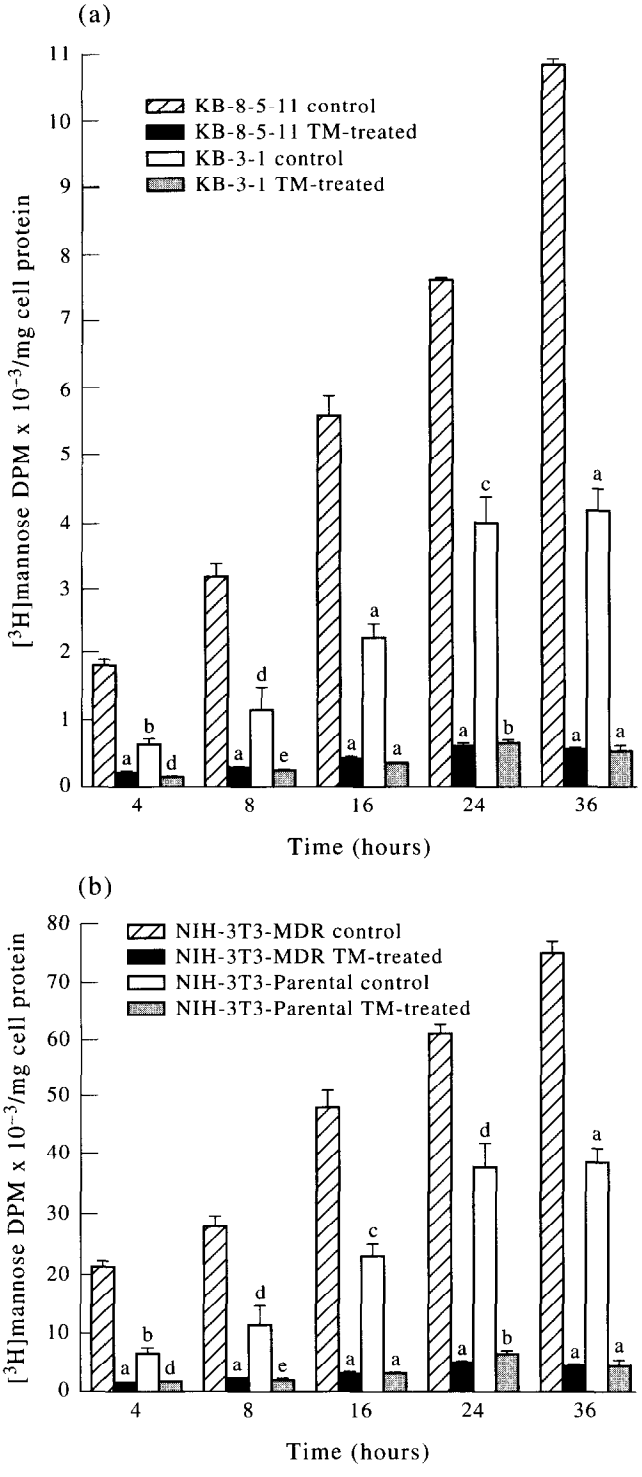

Figure 1. Effect of 5 μg/ml tunicamycin on the synthesis of mannosylglycoproteins in KB-8-5-11 and KB-3-1 cells (a), and in NIH-3T3-MDR and NIH-3T3-parental cells (b) measured by the incorporation of [^{3}H]mannose into trichloroacetic acid-precipitated cellular protein. Data represent means ± S.E.M. (n = 4). Lowercase letters above bars indicate two-tailed P values for the difference between control and TM-treated cells, and between untreated (control) drug-resistant and drug-sensitive cell lines as follows: $^aP \leq 0.0001$; $^bP \leq 0.0003$; $^cP \leq 0.0007$; $^dP \leq 0.0004$; $^eP \leq 0.004$.

MTT cytotoxicity assay. This decrease in cell survival was concentration dependent. The consequence of TM pretreatment of cells for 16–72 h was not toxic and completely reversible with time after its removal from the culture medium. Accordingly, a TM concentration of 5 μg/ml and conditions under which cell viability remained unaffected were chosen to test the outcome of antibiotic treatment on the sensitivity of drug-sensitive and drug-resistant cells to various anticancer drugs.

Tunicamycin decreased the IC_{50} for DOX, EPX, VCR and COL after 72 h drug treatment in all the cell lines, except in the KB-3-1 cell line for which the IC_{50}s for DOX and COL were increased, and the IC_{50} for EPX was not reduced significantly (Table 1). Sensitisation of NIH-3T3-MDR and KB-8-5-11 drug-resistant cells to DOX, EPX, VCR and COL by TM was considerably greater than in the corresponding parental cells. As a result, TM decreased the relative resistance of NIH-3T3-MDR and KB-8-5-11 drug-resistant cell lines to DOX, EPX, VCR and COL. Surprisingly, TM enhanced the toxicity of CPL (commonly regarded as a non-MDR-related drug) in NIH-3T3-MDR cells, but not in NIH-3T3-parental cells. The reversal of drug resistance in NIH-3T3-MDR and KB-8-5-11 by TM varied for the different anticancer drugs and there was no apparent consistency between the degree of resistance and fold reversal (Table 1). The sensitisation effect of TM was abolished after 24 h exclusion of the antibiotic from the culture media (data not shown).

Vincristine transport and retention

Vincristine uptake and efflux curves for the human epidermoid carcinoma KB-3-1 parental cell line and its multidrug-related derivative, KB-8-5-11, are shown in Figure 2. Uptake of VCR by KB-3-1 cells was unaffected by the presence of TM (Figure 1a), but efflux was increased for the 60-, 80- and 100-min postincubation periods (Figure 1b). Tunicamycin also failed to alter the uptake of VCR by KB-8-5-11 cells, except at 120 min ($P = 0.01$; Figure 1a). The typical rapid efflux of VCR was significantly impaired in KB-8-5-11 cells following pretreatment and co-incubation with TM (Figure 1b).

The effects of TM on the uptake and efflux of VCR in cultures of NIH-3T3-parental and NIH-3T3-MDR cells are shown in Figure 3. Pretreatment of NIH-3T3-parental cells with TM did not significantly alter their ability to accumulate VCR (Figure 3a). Similarly in NIH-3T3-MDR cells, TM treatment had no effect on the level of VCR accumulation (Figure 3a). NIH-3T3-MDR cells also accumulated less drug than their drug-sensitive parental cells (Figure 3a). In both cell lines, uptake displayed a consistent biphasic course with a rapid initial component and a subsequent linear component (the drug-cell system reaches a steady state within 20–30 min). Efflux curves for NIH-3T3-parental and NIH-3T3-MDR cells are depicted in Figure 3a. Tunicamycin had no effect on the efflux of VCR from NIH-3T3-parental cells. By contrast, VCR efflux was significantly reduced ($P < 0.05$ at all postincubation times) when *MDR*1-transfected NIH-3T3 cells were treated with TM. It is noteworthy that the NIH-3T3-MDR cell line contained significantly less VCR after an initial 1-h preloading period when compared with its parental cell line (Figure 3b). This further supports the notion that MDR is

Table 1. *The effects of tunicamycin on drug toxicity* in drug-sensitive (NIH-3T3-P and KB-3-1) and drug-resistant (NIH-3T3-MDR and KB-8-5-11) cells*

Anticancer drug	Cell line/ variant	IC_{50}† ± S.E.M. ($n = 8$) Control	TM-treated	Two-sided *P*-values‡	Relative resistance§ without TM	with TM	Sensitisation ratio‖ (95% CI)	Fold reversal¶
Doxorubicin	NIH-3T3-P	0.33 ± 0.05	0.12 ± 0.02	0.0036			2.75 (1.82 to 5.0)	
	NIH-3T3-MDR	1.63 ± 0.05	0.20 ± 0.09	<0.0001	4.94	1.67	8.14 (5.56 to 12.5)	2.96
	KB-3-1	0.12 ± 0.01	0.29 ± 0.02	<0.0001			0.42 (0.29 to 0.55)	
	KB-8-5-11	5.58 ± 0.22	0.92 ± 0.05	<0.0001	46.5	3.17	6.08 (3.36 to 8.80)	14.67
Epirubicin	NIH-3T3-P	7.91 ± 0.26	3.43 ± 0.15	<0.0001			2.31 (2.12 to 2.50)	
	NIH-3T3-MDR	32.03 ± 1.26	0.74 ± 0.03	<0.0001	4.05	0.22	43.29 (33 to 50)	18.41
	KB-3-1	0.93 ± 0.34	0.38 ± 0.02	0.1504			2.45 (1.92 to 3.22)	
	KB-8-5-11	14.63 ± 1.01	2.86 ± 0.23	<0.0001	15.73	7.53	5.11 (4.54 to 5.55)	2.09
Vincristine	NIH-3T3-P	3.94 ± 0.51	2.66 ± 0.24	0.0493			1.48 (1.39 to 1.54)	
	NIH-3T3-MDR	31.02 ± 2.97	0.33 ± 0.01	<0.0001	7.87	0.12	94.33 (87 to 100)	65.58
	KB-3-1	2.19 ± 0.23	0.015 ± 0.002	<0.0001			143 (139 to 145)	
	KB-8-5-11	65.04 ± 8.82	0.18 ± 0.03	0.0002	29.69	12.00	352 (228 to 387)	2.47
Colchicine	NIH-3T3-P	3.39 ± 0.53	1.20 ± 0.09	0.0047			2.82 (2.43 to 3.33)	
	NIH-3T3-MDR	31.81 ± 3.72	0.51 ± 0.09	<0.0001	9.38	0.43	62.40 (50 to 100)	21.81
	KB-3-1	0.41 ± 0.05	0.93 ± 0.16	0.0146			0.44 (0.36 to 0.52)	
	KB-8-5-11	39.96 ± 6.09	0.11 ± 0.007	0.0003	97.50	0.12	366 (358 to 374)	812.50
Cisplatin	NIH-3T3-P	2.84 ± 0.38	2.65 ± 0.32	0.7083			1.07 (0.35 to 3.25)	
	NIH-3T3-MDR	36.42 ± 4.27	0.34 ± 0.04	<0.0001	12.82	0.13	107 (105 to 109)	98.62

*Assessed by the MTT assay; †Drug concentration in μg/ml that decreased the final absorbance of reduced MTT to 50%, relative to the absorbance observed with cells not treated with drug (cf. *in vitro* cytotoxicity assays in Materials and Methods). Each IC_{50} value is the mean representative of one experiment out of three performed separately, all of which yielded comparable results. ‡Determined by the unpaired alternate *t*-test for Gaussian distributions with different standard deviations; §Calculated by dividing the IC_{50} for each drug in drug-resistant cells by that in drug-sensitive cells; ‖Ratio of IC_{50} (untreated, control)/IC_{50} (TM-treated); ¶Computed by dividing the relative resistance of drug obtained without TM by that obtained with TM. S.E.M., standard error of the mean; P, parental; MDR, multidrug-resistant.

associated with decreased intracellular accumulation of drug. Data obtained in separate experiments similar to those described for the efflux studies on the retention of VCR in various drug-sensitive and drug-resistant cell lines in response to TM treatment are summarised in Figure 4. Tunicamycin increased the retention of VCR in KB-8-5-11 cells, in NIH-3T3-MDR cells (at post-drug-loading periods of 40, 60 and 80 min), but not in NIH-3T3-parental cells. By contrast, TM-treated KB-3-1 cells expressed a decreased VCR retention at 20- (marginally significant), 40-, 60- and 80-min postincubation periods. However, after 100 min, the retention levels of VCR in both control and TM-treated KB-3-1 cells were equal.

DISCUSSION

The effects of TM have been studied in various systems, primarily with the aim of elucidating the role of the carbohydrate moieties in biological processes. It has been suggested that TM may be selectively more toxic to transformed cells than to non-transformed cells, although this may not always be the case [25]. The inhibition of *in vitro* cell growth by TM is cell cycle-specific since only G_1-phase cells are blocked in their cell-cycle progression [26] Moreover, TM suppresses the activity of 3-hydroxy-3-methylglutaryl coenzyme-A reductase which catalyses the rate-limiting step in the biosynthesis of cholesterol and isoprenoid derivatives [27]. This raises the possibility that TM exerts its inhibitory effects on cell proliferation via the isoprene-synthetic pathway in parallel with its effects on asparagine-linked glycosylation [28, 29]. It is noteworthy that resistance to TM in Chinese ovary cells is also associated with gene amplification [30] comparable with that occurring in MDR cells [31]. These pathways may have experimental and clinical ramifications for the expression of cellular drug resistance.

The function of the oligosaccharide moieties of P-glycoprotein in MDR has not been evaluated adequately. Anthracycline resistance in HL-60 human promyelocytic leukaemia cells is associated with hypoglycosylation of cell-surface glycoproteins [32]. However, P-glycoprotein is not overexpressed in these cells. Tunicamycin has been shown previously to inhibit N-glycosylation of P-glycoprotein in drug-resistant cells without any effect on the expression of resistance [33]. Similarly, a glycosylation mutant of drug-resistant CH^RC5 cells has been found to retain its phenotype [34], implying that the oligosaccharide chains of P-glycoprotein are probably not involved in the expression of drug resistance. The fact that not all glycoproteins become unstable or non-functional in the unglycosylated form [18], would suggest that P-glycoprotein may be synthesised at normal rates, although deficient in carbohydrate, and still sustain its biological activity in MDR [35]. Differential glycosylation of the *MDR*1 gene product and the biosynthesis of heterogeneous forms of MDR-associated glycoproteins in different cell lines [36] indicate that complex genetic regulation may determine cell sensitivity to cytotoxic drugs. Recently, it has been demonstrated that inhibition of post-translational processing (glycosylation) of P-glycoprotein with TM results in increased daunorubicin accumulation [37]. This finding not only indicates a role for P-glycoprotein and cell-surface carbohydrate in mediating MDR [13], but also supports the concept that a reduction in P-glycoprotein efflux pump activity may be essential for reduced drug transport and/or increased drug sensitivity.

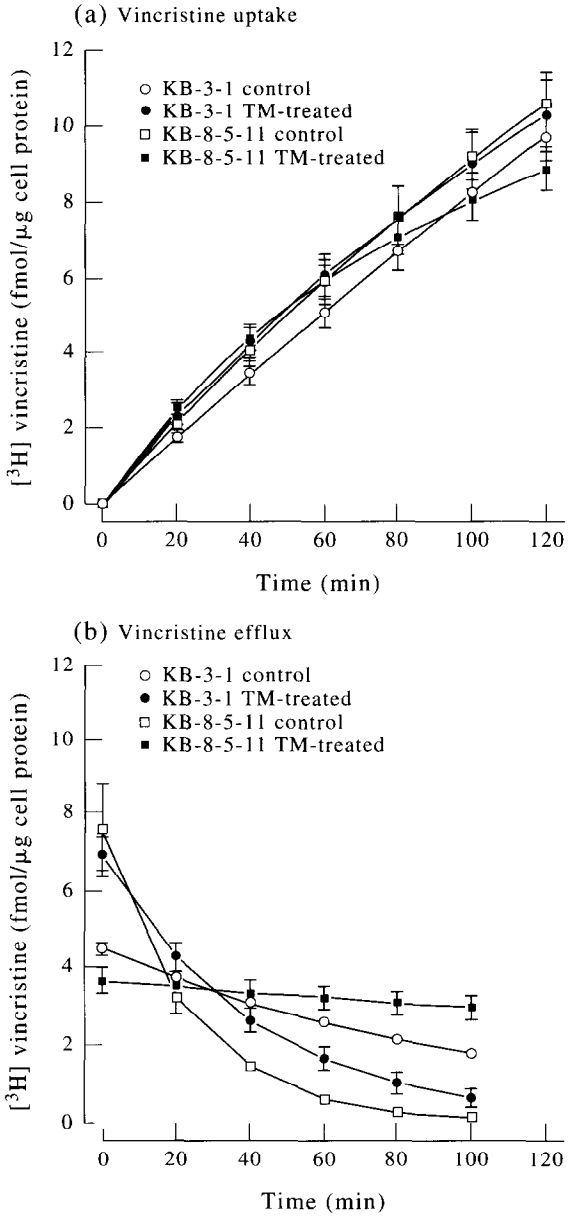

Figure 2. Time course of vincristine uptake and efflux in drug-sensitive KB-3-1 and multidrug-resistant KB-8-5-11 cells cultured in the absence or presence of TM following prior exposure to the antibiotic for 16 h. Values are means ± S.E.M. ($n = 4$). Where error bars do not overlap, Student's two-tailed P values are less than 0.05.

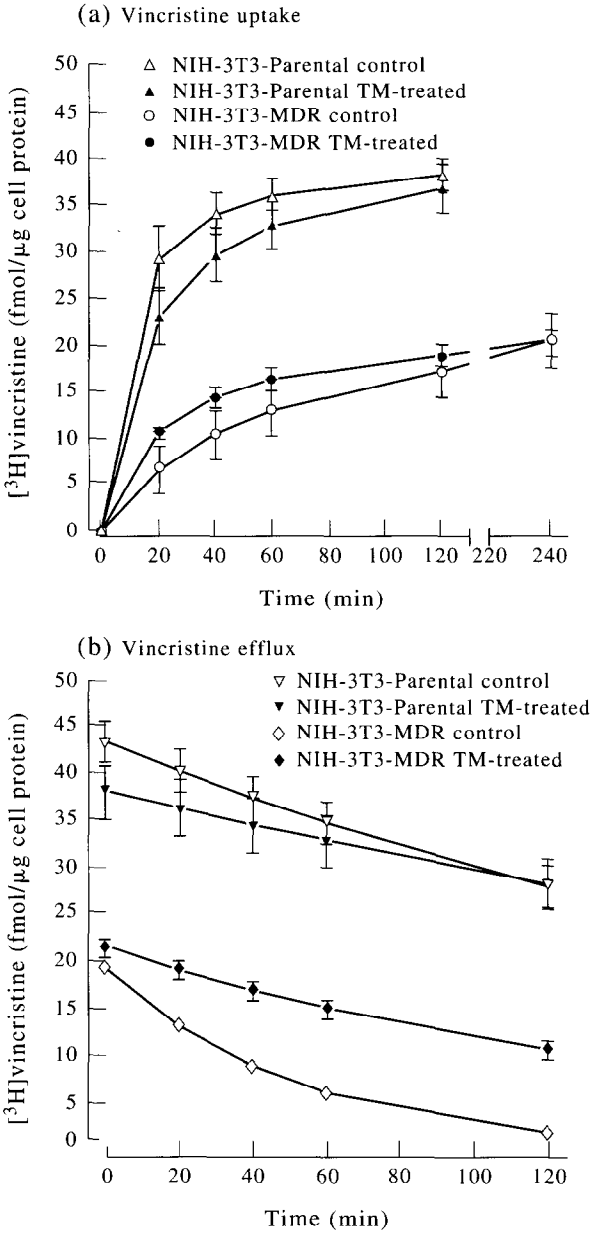

Figure 3. Time course of vincristine uptake (a) and efflux (b) in NIH-3T3-Parental and NIH-3T3-MDR cells cultured in the absence or presence of TM following a 16 h TM pretreatment. Details are described in the legend to Figure 2. Error bars are shown on the figure when larger than the plot symbols.

The results of the present study indicate that TM augments the susceptibility to anticancer agents of drug-resistant cell lines to a greater extent than their respective parental controls. The uptake of VCR was not affected in the various cell lines following prior exposure to and co-incubation with TM. Cellular VCR loading was significantly greater in NIH-3T3-parental cells compared with their multidrug-resistant counterparts. The decreased VCR accumulation in NIH-3T3-MDR cells can be ascribed to enhanced VCR efflux, since these cells overexpress P-glycoprotein. However, no differences in the levels of VCR accumulation could be demonstrated between KB-3-1-parental and KB-8-5-11-MDR cells. In KB-3-1 cells, conflicting and disparate responses to TM exposure were observed, including increased IC_{50} values for DOX and COL, unchanged IC_{50}s for EPX, and reduced VCR retention, suggesting that comparison of the effects of TM between drug-sensitive and drug-resistant cell lines should be made with caution and that such effects may be relatively non-specific and thus not widely applicable. Nevertheless, a reproducible association was found between treatment of MDR cells (NIH-3T3-MDR and KB-8-5-11) with TM and reduced VCR efflux with resultant increase in VCR retention. Tunicamycin did not alter drug efflux and consequently VCR retention in NIH-3T3-parental cells.

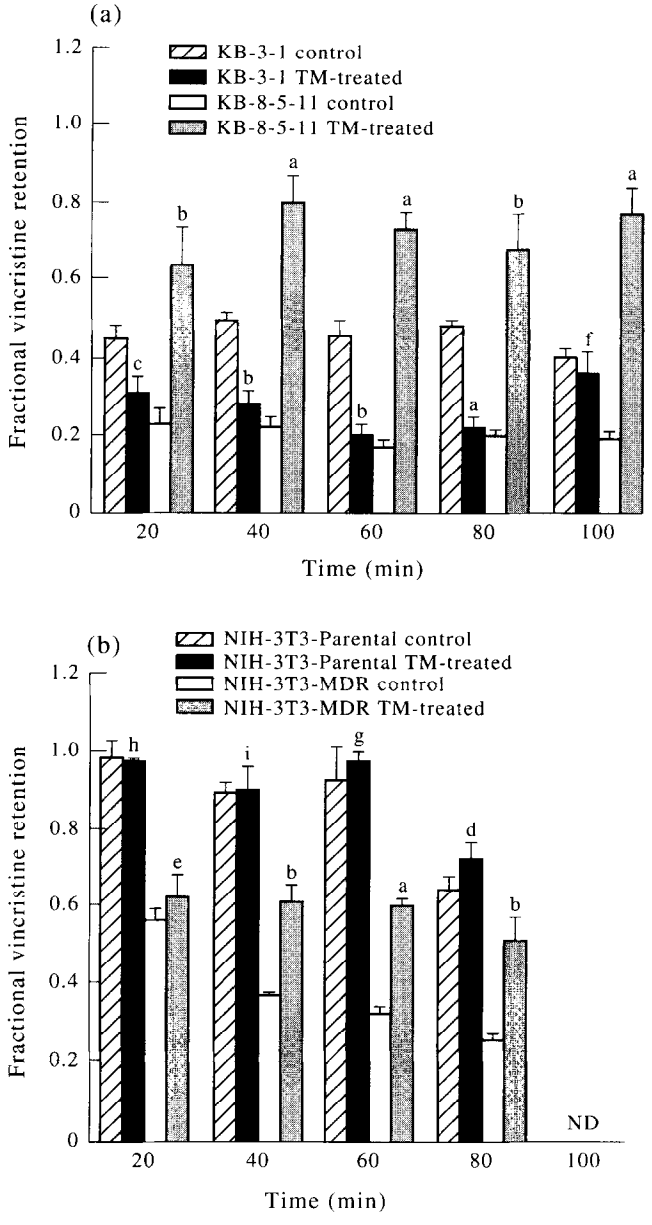

Figure 4. Comparison of the effects of TM on the retention of vincristine in KB-8-5-11 and KB-3-1 cells (a), and in NIH-3T3-MDR and NIH-3T3-parental cells (b) at different time intervals after a 1-h preloading period with the drug and subsequent exposure to drug-free medium. Values are means ± S.E.M. ($n = 4$). Lowercase letters above bars indicate two-tailed P values for the difference between control and TM-treated cells as follows: $^{a}P \leqslant 0.0003$; $^{b}P \leqslant 0.008$; $^{c}P = 0.053$; $^{d}P = 0.174$; $^{e}P = 0.381$; $^{f}P = 0.538$; $^{g}P = 0.617$; $^{h}P = 0.835$; $^{i}P = 0.881$.

Overall, the results show that TM enhances VCR retention in MDR cell lines by limiting drug efflux. KB-8-5-11 and NIH-3T3-MDR cells appear to be more responsive to TM pretreatment, possibly due to the common inhibitory effects of the antibiotic on the expression of glycoproteins. It is conceivable that TM induces lesions in the oligosaccharide moiety of P-glycoprotein in the MDR cell lines which contributes to the increased VCR retention that is coupled to decreased efflux of the vinca alkaloid. Alternatively, TM may exert its effect by independent modulation of efflux pump activity. Transient inhibition of glycoprotein synthesis during TM exposure may result in alterations of VCR efflux and retention observed in the MDR cell lines. The extent to which TM may modify the molecular conformation of P-glycoprotein synthesised *de novo*, and its ability to engage in intermolecular interactions such as drug recognition, binding and efflux is not known.

To address this concern, further investigations into these and distinct effects of TM on the sensitivity of cancer cells to chemotherapeutic drugs in relation to their proliferative state should be considered. Rapidly dividing tumour cells will be highly responsive to drugs toxic in the S-phase of the cell cycle [38]. Correspondingly, it has been demonstrated previously that cells treated with TM, an inhibitor of N-linked glycosylation, fail to enter the S-phase [39]. This observation raises the tangible question of whether N-linked glycosylation required for initiation of DNA synthesis is coupled to the expression of high-glycosylation required for initiation of DNA synthesis or is coupled to the expression of high-molecular-weight (90–240 kDa) type glycoproteins, e.g. growth factor receptors. Accordingly, this relationship may be a useful indicator of TM-induced alterations in the 170–190 kDa P-glycoprotein frequently associated with multidrug resistance.

Although the results of this study do not provide a comprehensive answer to the mechanisms by which TM affects the MDR phenotype, they may be useful in assessing the effects of glycosylation on the properties of P-glycoprotein and its interaction with cytotoxic drugs. In the broader sense, it is tentative that glycosylation regulates drug efflux in drug-resistant cells through an, as yet, undetermined mechanism. Tunicamycin may also accumulate within cells and serve as a substrate for P-glycoprotein. Such a possibility can be studied specifically by measuring the intracellular distribution of radiolabelled antibiotic [40] in drug-sensitive and drug-resistant cells. Isobologram analysis could also be performed to determine cytotoxic potentiating effects of TM on anticancer drugs. Recently, inhibition of N-linked glycosylation by TM has been shown to affect organic cation transport across the brush border membrane of opossum kidney cells [41]. This information, in association with the observation that P-glycoprotein binds certain organic chemicals in the adrenal gland and kidney, and presumably also functions in the excretion of these compounds [42], and the knowledge that P-glycoproteins from KB-C2 cells, kidney and adrenal gland exhibit different lectin binding capacities [14], may be applied effectively in strategies to overcome MDR. However, the potential use of TM as an MDR modifier must await clarification of the precise action of this antibiotic on P-glycoprotein.

The finding that TM significantly enhances the cytotoxicity of CPL in NIH-3T3-MDR cells is puzzling, but also of particular interest, since it is generally believed that the mechanism of CPL refractoriness differs from that of MDR [43]. The platinum complexes, e.g. *cis*-DDP (*cis*-dichlorodiaminoplatinum II), represent an important class of cytotoxic drugs generally capable of forming cross-links in DNA critical to their cytotoxicity [44, 45]. Furthermore, it has been suggested that acquired cellular non-responsiveness to CPL may be associated with increased gene-specific DNA repair of interstrand cross-links [46, 47]. Although *cis*-DDP-resistant cells also exhibit decreased uptake of platinum

compounds [48], it is well established that the reduced accumulation is not due to *MDR*1 gene expression [49]. Cell lines selected for resistance to drugs in the MDR class (e.g. vincristine, vinblastine, doxorubicin, colchicine and actinomycin D) usually express cross-resistance to other drugs in the same class [50], but not to DNA intercalating agents (e.g. methotrexate, cisplatin, nirosoureas). Similarly, a DOX-resistant multiple myeloma cell line displayed a sensitivity pattern to the nitrosureas (streptozotocin, STZ; 1,3-bis[chloroethyl]-1-nitrosourea, BCNU) identical to its drug-sensitive parental cell line [51], hence implying that cells sensitive to 'MDR-related' drugs are not resistant to nitrosoureas.

In general, drugs which modify the MDR phenotype do not show a sensitising effect to cisplatin [52], although some resistance modifiers, such as nifedipine, may synergistically enhance the antitumour effect of cisplatin on MDR cells [53]. Alternatively, cell lines vary in their ability to replace DNA lesions, and their repair capacity may be intrinsically related to cellular accumulation and sensitivity to antitumour drug [54]. It is probable that P-glycoprotein-mediated MDR and *cis*-DDP refractory phenotypes may coexist in certain tumour types [55, 56]. Therefore, compounds which will induce DNA strand breaks and inhibit DNA repair could be of particular value in reversing such drug resistance mechanisms [47, 54, 57]. The cytotoxicity and DNA damage induced by anthracyclines (e.g. doxorubicin and daunorubicin), DNA intercalating agents and epipodophyllotoxins (e.g. etoposide and teniposide) appear to be mediated by topoisomerase II [58]. Recent studies have implicated alterations in activity and function of this enzyme as an alternative mechanism of MDR [59]. Although topoisomerase II is a principal determinant of cell sensitivity to DNA poisons, other changes involved in the regulation of enzyme function and/or in the cellular processing of drug-induced DNA damage may be critical in determining disparate cell responses to antitumour agents. The precise nature of these alterations in MDR has yet to be established. However, several studies have demonstrated that the design of well-defined DNA intercalators and drugs is both feasible and potentially useful to overcome atypical MDR [60, 61]. Similar interpretations applicable to the role of N-glycosylation in tumour cell-cycle progression and sensitivity to anticancer drugs may lead to new directions in overcoming multiple forms of drug resistance.

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