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RESISTANCE TO TETRACYCLINE, A HYDROPHILIC ANTIBIOTIC, IS MEDIATED BY P-GLYCOPROTEIN IN HUMAN MULTIDRUG-RESISTANT CELLS

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Summary: Two multidrug-resistant human leukemic CCRF-CEM sublines (CEM/VCR R and CEM/VLB_{100}) were significantly more resistant to tetracycline, a hydrophilic antibiotic, than parental cells ($P < 0.001$). Verapamil and cyclosporin A completely reversed tetracycline resistance in CEM/VCR R cells, which also accumulated and retained significantly less [^{3}H]tetracycline than CCRF-CEM cells. Like verapamil, addition of tetracycline to CEM/VCR R cells which had achieved steady-state vincristine levels resulted in augmented vincristine accumulation. [^{3}H]Azidopine photoaffinity labelling of CEM/VCR R membrane proteins was inhibited by tetracycline in a dose-dependent manner. Although drugs associated with the multidrug-resistance phenotype are typically hydrophobic compounds, these data suggest that resistance to tetracycline, despite its hydrophilic nature, is mediated by P-glycoprotein in these cell lines.

The phenomenon of multidrug resistance (MDR), in which selection for resistance of cells to certain "natural product" drugs results in cross-resistance to other structurally unrelated compounds, has been well characterized (1-3). The MDR phenotype has been associated with decreased accumulation of drug due to the over-expression of a membrane-associated protein, P-glycoprotein, which acts as an energy-dependent efflux pump (4,5). A number of agents, exemplified by verapamil and cyclosporin A, have been described which are capable of reversing MDR *in vitro* by binding to P-glycoprotein (6,7).

Compounds associated with the P-glycoprotein-mediated MDR phenotype, despite little structural similarity, tend to be bulky, lipophilic molecules (8). Thus, both cytotoxic drugs and modulating agents which are apparently recognized and bound by P-glycoprotein (9,10) are lipid-soluble at physiological pH, as defined by oil/water partitioning (11,12). A recent brief report has suggested that tetracycline might be recognized by P-glycoprotein (13). In view of the relative hydrophilicity of tetracycline, its oil/water partition coefficient ($\log_{10}$ P) being an order of magnitude lower than even the least lipophilic of the characterized substrates of

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Abbreviations: MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

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P-glycoprotein (11,14), this result was unexpected. We have therefore assessed the cytotoxicity of tetracycline in human MDR leukemic cells and demonstrated that these cells are significantly more resistant to this compound than parental, drug-sensitive cells. Further, we have investigated the mechanism of this resistance, and conclude that, despite the hydrophilic nature of tetracycline, resistance to this drug is mediated by P-glycoprotein.

MATERIALS AND METHODS

Chemicals. [^{3}H]azidopine (49 Ci/mmol), [^{3}H]vincristine (7.1 Ci/mmol) and non-radioactive azidopine were purchased from Amersham Australia; [^{3}H]tetracycline (0.5 Ci/mmol) from Dupont-NEN Research Products; vincristine and vinblastine sulphate from Eli Lilly; tetracycline hydrochloride from Boehringer-Mannheim; verapamil hydrochloride from Knoll AG; Me_2SO and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) from Sigma, and thymidine from Calbiochem. Cyclosporin A, a generous gifts of Sandoz Inc., was dissolved in ethanol, to a final maximal ethanol concentration of 0.18%, which had no effect on cell survival.
Cell Culture. The human leukemic cell line CCRF-CEM (15) together with the "classic" MDR sublines CEM/VCR R (16) and CEM/VLB_{100} (17) were maintained in suspension culture as described previously (16). Cytotoxicity determinations were performed using the MTT assay, essentially as described (18), except that addition and solubilization of MTT were according to Park *et al.* (19), followed by addition of glycine buffer (20). Determination of ID_{50} values and statistical analysis was performed as described previously (21).
Drug Accumulation and Efflux Studies. Intracellular accumulation and retention of tetracycline (final concentration, 100 μM) were determined as described (16). To examine the effect of various compounds on vincristine accumulation, cell suspensions (50 ml) were incubated (37°C, 2 h) in the presence of 50 nM [^{3}H]vincristine, prior to the addition of verapamil (10 μM), tetracycline (4mM) or 0.9% NaCl. Incubations were terminated by removing duplicate aliquots (1ml) and treating them as described (16).
Photoaffinity Labelling of Cell Membranes. Membranes from $2x10^8$ cells were prepared as described (22), prior to photoaffinity labelling (50μg protein) with 0.05μM[^{3}H]azidopine (23), electrophoresis (24) and fluorography (20% 2,5-diphenyloxazole/ Me_2SO).

RESULTS

Two human leukemic MDR cell lines, CEM/VCR R and CEM/VLB_{100} were each more resistant to tetracycline than parental CCRF-CEM cells (Table 1). The relative resistance of these cell lines was, in each case, highly statistically significant. The two MDR cell lines did not differ significantly from each other in terms of their ID_{50} values for tetracycline ($P > 0.05$). The effects on tetracycline cytotoxicity of the MDR response modifiers, verapamil and cyclosporin A, were investigated in CEM/VCR R cells (Fig. 1). For comparative purposes, the effect of the modifiers on vincristine resistance was also determined. In the parent CCRF-CEM line, neither compound affected the cytotoxicity of either tetracycline or vincristine ($P > 0.05$; data not shown). In contrast, both verapamil and cyclosporin A significantly reduced the ID_{50} value not only of vincristine in the CEM/VCR R cells, but also of tetracycline ($P < 0.005$ in all cases). The effect of either modifier was to virtually eliminate tetracycline resistance, since the ID_{50} values for tetracycline in these cells in the presence of the modifiers were not significantly different from that of the parent cells.

When accumulation of tetracycline was compared in CEM/VCR R cells and parent CCRF-CEM cells, the resistant cells accumulated significantly less tetracycline than CCRF-CEM (Fig. 2). By one hour, both cell populations had virtually achieved steady state tetracycline

Table 1. Resistance to tetracycline in CCRF-CEM sublines

Cells	ID_{50} [a]		Relative Resistance [c]	P [d]
	Log [Tetracycline] [b] ±S.E.	μM		
CCRF-CEM	-4.60 ± 0.02	25.1	-	-
CEM/VCR R	-4.21 ± 0.03	61.3	2.4	<.001
CEM/VLB_{100}	-4.11 ± 0.04	78.1	3.1	<.001

a Defined as the drug concentration which reduces absorbance, in the MTT assay, to 50% of that in the control wells.
b Calculated from at least 4 replicate assays.
c Determined by dividing the mean ID_{50} for the resistant subline by the mean ID_{50} of the parent CCRF-CEM cells.
d Determined by comparing ID_{50} values for the sublines shown with the ID_{50} value of the parent CCRF-CEM cells.

concentrations, and the differential in tetracycline accumulation between the two lines was maintained following a further hour of incubation. The amount of tetracycline effluxed by each of the cell populations following a 60 min preincubation was determined (Table 2). Intracellular levels of tetracycline were consistently lower in CEM/VCR R than CCRF-CEM cells, and by one hour, the resistant cells had effluxed a significantly greater proportion (84%) of their

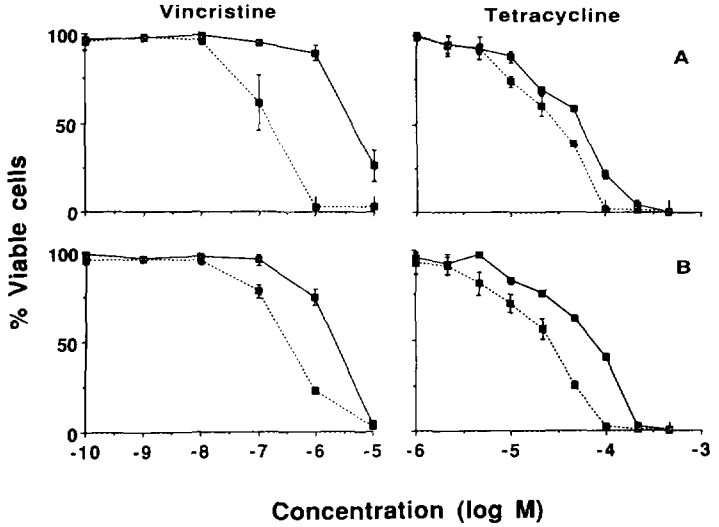


Figure 1. Effect of response modifiers (A) verapamil (10 μM) or (B) cyclosporin A (2.1 μM) on cytotoxicity of CEM/VCR R cells. Cells (7,500/ microtitre well) were exposed to vincristine or tetracycline for 72 h in the presence (- -) or absence (—) of the modifiers. Mean numbers of viable cells (± S.E.) are expressed as a percentage of cell growth in the presence of the modifier but the absence of either vincristine or tetracycline.

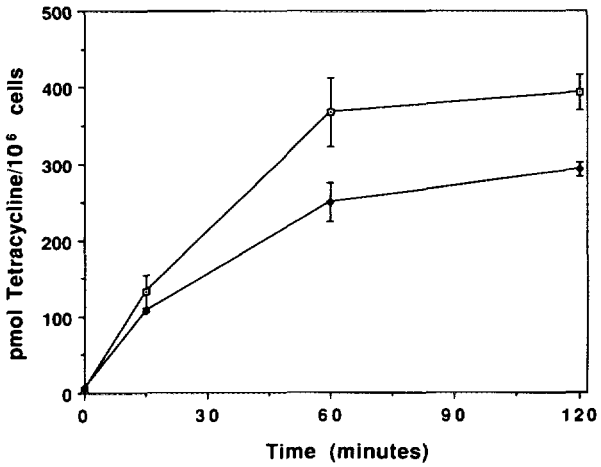


Figure 2. Accumulation of [^{3}H]tetracycline by CCRF-CEM (□) and CEM/VCR R (◆) cells. Cells (5 x 10^6/ml) were incubated at 37°C in RPMI 1640 containing 100μM [^{3}H]tetracycline, and at the times indicated, intracellular radioactivity was determined (16). Each point represents the mean (± S.E.) of duplicate determinations from at least 3 experiments run on separate days.

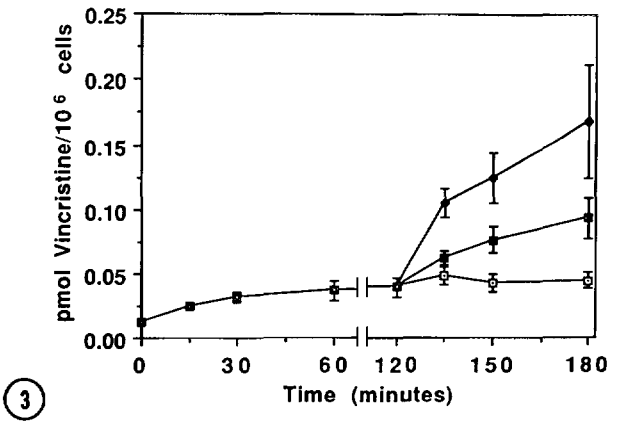
[^{3}H]tetracycline. To determine whether the enhanced efflux of tetracycline in the MDR cells was likely to be mediated by P-glycoprotein, we examined the effect of tetracycline on vincristine accumulation (Fig. 3). As anticipated, addition of verapamil to CEM/VCR R cells which had achieved a low, steady-state level of vincristine, resulted in a rapid increase in their intracellular drug level, with a 3.7-fold increase in vincristine accumulation being achieved 60 min following

Table 2. Efflux of tetracycline from parent and multidrug-resistant CCRF-CEM cells

Efflux Time (min)	CCRF-CEM		CEM/VCR R	
	Intracellular Tetracycline (pmol/10^6 cell)	%Effluxed	Intracellular Tetracycline (pmol/10^6 cell)	%Effluxed
0	297 ± 19 [a]	-	232 ± 13	-
15	226 ± 16	23.7 [b]	133 ± 7	42.3
30	144 ± 12	51.1	91 ± 8	60.4
45	121 ± 11	59.0	68 ± 8	70.6
60	102 ± 10	65.6	38 ± 3	83.5

[a] Mean ± S.E. of duplicate determinations from three experiments run on separate occasions.

[b] Proportion of tetracycline effluxed expressed as a percentage of the zero-time tetracycline concentration.



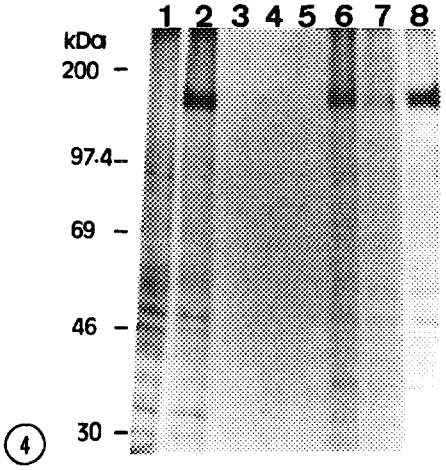


Figure 3. Effect of tetracycline or verapamil on net accumulation of [^{3}H]vincristine by CEM/VCR R cells. Cells were incubated at 37°C in the presence of 50nM [^{3}H]vincristine (■) for 2 hr prior to the addition of verapamil (10μM, ◆), tetracycline (4mM, ■) or 0.9% NaCl (□). Intracellular radioactivity of aliquots removed at various times over the next 60 min was determined as described (16). Each point represents the mean (± S.E.) of duplicate determinations from at least 3 experiments run on separate days.

Figure 4. Photoaffinity labelling by [^{3}H]azidopine of plasma membranes from drug-sensitive CCRF-CEM cells (lane 1) and drug-resistant CEM/VCR R (lanes 2-8) cells. Photoaffinity labelling (23) was carried out in the absence of inhibitors (lane 2) or in the presence of 100μM or 1mM tetracycline (lanes 6 and 7), 200μM azidopine (lane 5), or 2mM thymidine (lane 8), prior to 7% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and fluorography. Photolabelling of CEM/VCR R membranes was not observed in the absence of UV irradiation (lane 4) or when the [^{3}H]azidopine was UV irradiated prior to incubation with membranes (lane 3). Equal loading of protein in all lanes was confirmed by staining of gels with Coomassie Blue.

addition of the modifier. Addition of tetracycline to the cells similarly augmented vincristine accumulation (2.1-fold increase). In the absence of added modifiers, the steady-state level of vincristine remained unchanged. Neither verapamil nor tetracycline perturbed vincristine accumulation in the drug-sensitive CCRF-CEM cells (data not shown).

Photoaffinity labelling of plasma membrane proteins by [^{3}H]azidopine (Fig. 4) revealed a 170 kDa radiolabelled protein in membranes from CEM/VCR R (lane 2), but not CCRF-CEM (lane 1) cells. Increasing concentrations of unlabelled tetracycline progressively reduced radiolabelling of this protein, indicating the ability of tetracycline to compete for azidopine binding. Densitometric scanning of the fluorographic bands indicated that 100μM and 1mM tetracycline inhibited azidopine binding by 41% and 84%, respectively (lanes 6 and 7). Still higher concentrations of tetracycline completely eliminated photoaffinity labelling (data not shown). The specificity of the effect of tetracycline on the photoaffinity labelling reaction was confirmed by demonstrating that high concentrations (2mM) of another hydrophilic compound, thymidine, failed entirely to compete for photoaffinity labelling by azidopine (lane 8). When photolabelling was performed in the presence of excess unlabelled azidopine (lane 5), in the absence of UV irradiation (lane 4) or if the [^{3}H]azidopine was UV irradiated prior to incubation with membranes (lane 3), photolabelling failed to occur. These experiments were repeated using

plasma membranes isolated from the highly multidrug-resistant human adenocarcinoma cell line KB-V1(25), and essentially identical results were obtained (data not shown).

DISCUSSION

The octanol/water partitioning system has over the past 25 years been used to ascertain the $\log_{10}$ P values of over 15,000 compounds and is now a widely used standard of hydrophobicity [26]. Almost all drugs against which MDR cells are cross-resistant, and virtually all compounds capable of reversing MDR, are large, hydrophobic, cationic compounds, which are lipid-soluble at physiological pH, as defined by their octanol/water partition coefficients ($\log_{10}$ P, pH 7.4) of greater than 0.1 (11,12,27). The negative $\log_{10}$ P value of tetracycline ($\log_{10}$P = -1.44, pH7.5) (14) clearly indicates that it is a hydrophilic compound. Moreover, at physiological pH, it is either neutral or negatively charged (28). It would not, therefore, be anticipated that tetracycline would be a substrate for P-glycoprotein.

Hydrophilic compounds are presumably poor substrates for the multidrug transporter by virtue of not readily diffusing across membranes and hence not interacting with the lipid domains where P-glycoprotein is localised (29). However, Argast and Beck (30) have provided direct evidence that tetracycline, despite its hydrophilic character, can indeed diffuse through phospholipid bilayers. Although the nature of tetracycline transport across eukaryotic membranes has not been investigated in detail (31), similar diffusion of tetracycline across the mammalian plasma membrane would provide the opportunity for this compound to interact with P-glycoprotein.

While it is apparent that anionic hydrophilic compounds are not the preferred substrates of P-glycoprotein, we present, in this study, several lines of evidence in support of the proposition that tetracycline resistance in MDR cells is indeed mediated by P-glycoprotein. In addition to demonstrating resistance to tetracycline in two human leukaemic MDR cell lines known to express P-glycoprotein (16,17), we confirm that the resistance is completely reversed by verapamil and cyclosporin A, potent modulators of P-glycoprotein-mediated MDR (32,33). Moreover, we found evidence for reduced tetracycline accumulation and enhanced efflux of tetracycline in the MDR cells, and showed that tetracycline is capable of augmenting vincristine accumulation in these cells. Finally, tetracycline competes in a dose-dependent manner for binding of azidopine to a 170 kDa membrane protein present in the membranes of two independent MDR cell populations. Since azidopine has previously been shown to covalently bind, upon photoactivation, specifically to P-glycoprotein (23), these data collectively suggest that tetracycline is a substrate for P-glycoprotein, and as such, provide the first evidence that P-glycoprotein can bind and/or transport a hydrophilic compound.

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