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Interaction of multidrug-resistant Chinese hamster ovary cells with the peptide ionophore gramicidin D

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Abstract

A major form of multidrug resistance results from the overexpression of P-glycoprotein, a 170 kDa membrane protein. Multidrug resistant (MDR) Chinese hamster ovary (CHO) cells and *mdr1* transfectants displayed cross-resistance to the channel-forming peptide ionophore gramicidin D, which was reversed by various chemosensitizers, thus directly implicating P-glycoprotein as the mediator of resistance. However, gramicidin D was not able to inhibit [^{3}H]azidopine photolabelling of P-glycoprotein. MDR cells were not resistant to other pore-forming ionophores, but showed a modest level of cross-resistance to the mobile ionophore valinomycin. There was no difference in ^{125}I-gramicidin D uptake by resistant and sensitive cells. Resistant cells showed lower $^{86}Rb^+$ uptake, relative to the drug-sensitive parent. Addition of GmD increased both the rate and the level of $^{86}Rb^+$ uptake in sensitive cells, but had no effect on MDR cells. MDR cells also showed much lower rates of gramicidin D-dependent $^{86}Rb^+$ efflux than sensitive cells, and this was greatly increased by verapamil. These results suggest that P-glycoprotein interferes with the formation of ion-conducting gramicidin D channels. In contrast, valinomycin had the same effect on gramicidin D-dependent cation efflux in MDR and sensitive cells. Gramicidin D is thus unique among the ionophores in being a substrate for P-glycoprotein, which appears to greatly reduce the formation of active dimeric channels in the plasma membrane of MDR cells.

Key words: P-glycoprotein; Glycoprotein; Gramicidin D; Ionophore; Chemosensitizer; Cation efflux; Valinomycin

1. Introduction

Multidrug resistance continues to be a major impediment to the successful treatment of many human tumours by chemotherapy. The expression level of a 170 kDa integral membrane glycoprotein called P-glycoprotein (Pgp) has been shown to be correlated with the MDR phenotype (for reviews, see Refs. 1 and 2). The best evidence for its direct involvement in mediating MDR comes from transfection studies which demonstrated that the introduction of cDNA encoding Pgp conferred the MDR phenotype on drug-sensitive cells [3,4]. Sequence analysis of *mdr* genes [5,6], and numerous drug transport and binding experiments on MDR cells and plasma membrane vesicles (e.g., [7,8]), indicated that Pgp has all the features of a membrane-bound transporter that binds directly to hydrophobic drugs and utilizes ATP to transport them from the cell. Sequence comparisons have shown that Pgp is a member of a large family of membrane transporters known as the ABC (ATP-binding cassette) superfamily [9], or traffic ATPases [10].

Despite these observations, there are many aspects of the MDR phenotype which are not adequately explained by this model. Multidrug resistance has been shown to arise in the absence of Pgp overexpression in some cell lines (e.g., [11]), while electrophoretically distinct proteins have been observed to be overex-

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Abbreviations: CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; Gm, gramicidin; GmD, gramicidin D; MDR, multidrug resistant; α-MEM, alpha minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; Pgp, P-glycoprotein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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pressed in the plasma membrane of other cell lines [12]. Many intracellular effects have also been observed to correlate with the MDR phenotype, including altered DNA strand breakage by DNA topoisomerases [13], altered cytochrome *P*-450 [14] and glutathione *S*-transferase activity [15], and modified membrane structure [16] and composition [17]. Additional effects that appear at the level of the plasma membrane include reduced natural killer cell-mediated cell lysis of drug-resistant cells [18], elevated levels of membrane ether lipids [19] and triacylglycerols [20], and the resistance of MDR cells to the effects of ionophores such as GmD [21]..

Drugs transported by Pgp are, in general, lipophilic, and this has led to the proposal that the drug-binding site on Pgp may reside within the transmembrane domains [22]. This hypothesis is consistent with reports that these regions of Pgp are photolabelled by hydrophobic ligands [23], and site-directed mutations within transmembrane segments 6 and 11 result in changes in drug specificity [24,25]. The role of Pgp in normal tissues has yet to be elucidated and its physiological substrates have not been identified. However, the sequence homology of Pgp with proteins known to bind and export peptides, both in prokaryotes (the *Escherichia coli* hemolysin A exporter [26]; the oligopeptide permease of *Salmonella typhimurium* [27]) and eukaryotes (the yeast *ste6* a-factor exporter [28]; the Tap-1/Tap-2 putative peptide transporters in the endoplasmic reticulum [29]) suggests that peptides may also serve as Pgp substrates in vivo. Resistance to the hydrophobic peptide GmD is particularly intriguing, since it is thought to be cytotoxic by partitioning into the plasma membrane and forming cation-specific dimeric channels.

Because colchicine-selected CHO cells show high levels of cross-resistance to GmD, often higher than to certain anthracycline drugs [30,31], we were interested in determining the role of Pgp in resistance of this MDR cell model to the peptide ionophore. The results of the present study suggest that GmD may be a substrate for Pgp, which interferes with the ability of the peptide to form active cation channels in the plasma membrane of MDR cells.

2. Materials and methods

2.1. Cell lines

The colchicine-selected CHO cell lines used in this study were described previously [32]. Parental AuxB1, resistant CH^RA3, CH^RC5, and CH^RB30, revertant CH^RI10, and phytohemagglutinin-resistant CH^RPHA^R cell lines were cultured as monolayers in α-MEM supplemented with penicillin (1000 U ml^{-1}), streptomycin (1 mg ml^{-1}), 2 mM L-glutamine (all supplied by Gibco Laboratories, Burlington, Ontario) and 10% (v/v) heat-inactivated supplemented bovine calf serum (HyClone Laboratories, Logan, UT). The CH^RB30 cell line was maintained in 30 μg/ml colchicine. All cell lines were grown in 25-cm^2 culture flasks (Nunc) at 37°C in a humidified atmosphere of 5% CO_2.

CHO LR73 cells, murine *mdr1*-transfected LR73/1A cells, and mutant *mdr1*-transfected LR73/88-8 cells were obtained from Dr. P. Gros (McGill University), and were derived as described previously [33]. LR73/1A transfectants were grown in medium containing 0.1 μg/ml adriamycin. Western blot analysis, using the Pgp-specific mAb C219, indicated that Pgp expression levels in LR73/1A and LR73/88-8 plasma membrane vesicles were similar to those of the CH^RC5 line (Yu, X. and Sharom, F.J., unpublished data).

2.2. Growth inhibition of MDR cells

All compounds tested for their effect on CHO cell growth, except for modified GmD analogues (see below), were purchased from Sigma, (St. Louis, MO), and were used without further purification. They were all prepared as 20 or 200 mg/ml solutions in DMSO and stock solutions were diluted to 200 μg/ml or 2 mg/ml in α-MEM. The final DMSO concentration (1% v/v) had no effect on the growth rate or viability of any of the cell lines. The effect of various ionophores on the proliferation of MDR CHO cells was assayed by a modification of the MTT dye reduction assay, as described previously [34,35]. Cells were incubated with the test compound for 72 h before addition of MTT. All compounds were dissolved in DMSO and dispersed in culture medium containing 10% bovine calf serum, to reduce non-specific interactions with plastic surfaces. Relative cell growth was normalized by comparison to absorbance in the absence of ionophore, and the data were plotted as relative cell growth (% of control) vs. log ionophore concentration in μM (or nM, where appropriate). IC_{50} values were determined by interpolation from cytotoxicity plots. In general, all IC_{50} values for each compound were determined at the same time, so that they are internally consistent. Cross-resistance of each cell line towards a particular ionophore was calculated as the ratio of the IC_{50} value for the cell line relative to the AuxB1 (or LR73) parent.

Potentiation of GmD toxicity was determined in the presence of various chemosensitizers. Verapamil was used at a concentration of 20 μM. The IC_{50} of the other chemosensitizers was determined as above, and the concentrations selected for chemosensitization were half of the IC_{50} for AuxB1. Toxicity towards AuxB1 and CH^RC5 at the tested concentrations was minimal for all compounds tested. Relative cell growth was normalized by comparison to growth in the absence of

ionophore, but in the presence of the corresponding chemosensitizer. The chemosensitization index for each compound was calculated as the ratio of the IC_{50} value in the presence of chemosensitizer to that in its absence [36].

2.3. GmD and derivatives

GmD was *N*-deformylated by a modification of the method of Weiss and Koeppe [37]. Briefly, 2.5 g GmD was dissolved in 25 ml dry methanol, mixed with 25 ml 4 M HCl in dioxane (Aldrich, Milwaukee, WI), and stirred at 40°C for 1 h. The reaction mixture was evaporated to dryness and the resulting white solid was redissolved in a minimal amount of methanol and applied to a 200 ml Dowex 50W column. After extensive washing with methanol to remove unreacted GmD, the bound *N*-deformylated material was eluted with 2 M NH_4OH in methanol. Fractions were analyzed by thin-layer chromatography on plastic backed Kieselgel 60 plates (BDH, Toronto, Ontario), using the solvent system $CHCl_3$/ MeOH/ H_2O/ CH_3COOH (100:30:4:1, by vol.), and spraying with Ehrlich's reagent [38], and A_{280} was also measured. The pooled fractions were evaporated and lyophilized to give 1.5 g of *N*-deformylGmD.

Iodination of the single tyrosine residue of GmC, which makes up about 11% of the GmD preparation, was carried out using $Na^{125}I$ (ICN Biochemicals, St. Laurent, Québec) and Iodo-beads (Pierce, Rockford, IL). Briefly, 250 μCi of $Na^{125}I$ was added to 500 μg GmD in 500 μl methanol, along with one Iodo-bead, followed by incubation at room temperature for 30 min. Free iodide was removed by Sephadex LH-20 chromatography and GmD-containing fractions, as determined by thin-layer chromatography/gamma counting, were pooled and evaporated. Labelled GmD (specific activity around 450 mCi/mmol) was stored at 2 mg/ml in DMSO. The cytotoxicity of iodinated GmD was tested in AuxB1 and CH^RC5, and was not significantly different from that of the parent compound.

2.4. Uptake of ^{125}I-GmD by MDR CHO cells

Cells were harvested by trypsinization, and incubated in α-MEM for 2 h at 37°C before use. AuxB1 or CH^RC5 cells were suspended at $4 \cdot 10^6$ cells/ml in α-MEM/10% serum. For determination of time-dependent GmD uptake, 400 μl of 10 μM ^{125}I-GmD was added to 400 μl of cell suspension and the mixture was agitated at 37°C. At various times, cells were removed, layered over ice-cold 1 M sucrose/Dulbecco's PBS, and centrifuged at $15000 \times g$ for 1 min to remove unbound ionophore. Tubes were rapidly frozen in liquid nitrogen and the tips containing the cell pellet were sliced off and counted. Control samples contained ^{125}I-GmD, but no cells, to correct for GmD that sedimented through the gradient. These background values were very small, and were subtracted from the experimental data. For concentration-dependent uptake, ^{125}I-GmD solutions were prepared by serial dilution of a 5 μM stock solution. Cells and ionophore were agitated at 37°C for 40 min in medium and processed as above.

2.5. Uptake and efflux of $^{86}Rb^+$ by MDR cells

Cells were loaded with $^{86}Rb^+$ by addition of 1 μCi/ml $^{86}Rb^+$ in α-MEM/10% serum, followed by agitation at 37°C for 2 h. Cells were then rapidly washed, resuspended in a suitable volume of medium, and added immediately to 400 μl α-MEM/10% serum containing various concentrations of GmD, with or without 20 μM verapamil. At various times, aliquots were removed and the cells rapidly centrifuged through ice-cold 1 M sucrose, as described above. Efflux data were fitted to a first-order exponential rate equation using the program ENZFITTER (BioSoft), and half-times for cellular $^{86}Rb^+$ release were calculated by the program. $^{86}Rb^+$ uptake was assayed by adding 400 μl of cell suspension in medium to a 400 μl aliquot of medium containing 1 μCi/ml $^{86}Rb^+$ (2–40 nmol Rb^+ per assay), in the presence of GmD. At various times, aliquots of cells were removed and processed as above.

2.6. [^{3}H]Azidopine photoaffinity labelling

Plasma membrane vesicles were isolated from CH^RC5 cells, as described by Doige and Sharom [39]. Vesicles were thawed, centrifuged at $100000 \times g$ for 10 min in a Beckman Airfuge, and resuspended in buffer (10 mM Tris, 0.25 M sucrose, 4% v/v DMSO (pH 7.4)) containing 1 μg/ml aprotinin, leupeptin, and pepstatin A, and 25 μg/ml phenylmethylsulfonyl fluoride (all from Sigma). Photolabelling was then carried out, according to Safa et al. [40,41], in the presence of various concentrations of ionophores. Membrane vesicles were then analyzed by SDS-PAGE, followed by fluorography.

3. Results

3.1. Cross-resistance of MDR cells to ionophores

GmD was one of the first compounds to be identified as being part of the MDR spectrum of drugs to which MDR cells were cross-resistant [30]. We have used a modified MTT cytotoxicity assay to investigate GmD resistance in MDR CHO cells selected for colchicine resistance. The level of resistance to this

ionophore was indeed high; 30-fold for the CH^RC5 cell line (Fig. 1A and Table 1), and 38-fold for CH^RB30 (data not shown). In the series of cell lines tested, the degree of GmD resistance was correlated with Pgp expression levels in the plasma membrane, as shown by Western blot analysis with the monoclonal antibody C219 [39]. The GmD concentration required to inhibit growth of the AuxB1 parent was high (~ 0.5 μM), much larger than that necessary to increase K^+ permeability in erythrocytes [42], uncouple oxidative phosphorylation in intact mitochondria [43,44], or modify the transmembrane electrical potential of thylakoid membranes [45]. The revertant cell line CH^RI10 [46], which had barely detectable levels of Pgp (Loe, D.W. and Sharom, F.J., unpublished data), showed levels of sensitivity similar to the AuxB1 parental line (Table 1). CH^RPHA^R cells [31], which express underglycosylated Pgp, showed levels of GmD resistance similar to CH^RC5 (data not shown), indicating that Pgp oligosaccharides do not play a role in resistance to the ionophore.

mdr1-transfected LR73/1A cells acquired levels of GmD cross-resistance greatly exceeding those of the CH^RC5 or CH^RB30 lines (Fig. 1B, Table 2), and substantially higher than the levels of resistance to other classical MDR spectrum drugs, such as colchicine,

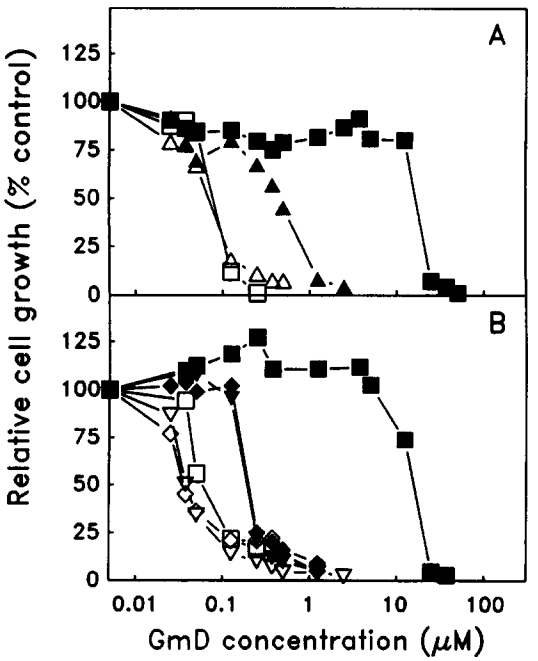

Fig. 1. Effect of GmD on the growth in vitro of multidrug resistant CHO cell lines. (A) CH^RC5 (■,□) and the drug-sensitive parent AuxB1 (▲,△). (B) murine *mdr1*-transfected LR73/1A (■,□), LR73/88-8 mutant *mdr1*-transfected (◆,◇), and drug-sensitive parent LR73 (▼,▽). Closed symbols indicate experiments conducted in the presence of GmD only, while the corresponding open symbols indicate experiments conducted in the presence of GmD and 20 μM verapamil. Data points represent means ($n = 3$). Limits of error (S.E.) fall within the symbols.

Table 1
Resistance and sensitivity of multidrug-resistant CHO cell lines to GmD and other peptide and non-peptide ionophores

Compound	IC_{50} (μM) [a]		
	AuxB1	CH^RC5	CH^RI10
Colchicine	0.06 (1.0) [b]	11.3 (190)	0.10 (1.7)
+Vrp	0.02 (3.0) [c]	0.75 (15)	0.025 (4)
GmD and derivatives			
GmD	0.53 (1.0)	15.8 (30)	0.89 (1.7)
+Vrp	0.095 (5.6)	0.16 (99)	0.23 (3.9)
DeformylGmD	65.8 (1.0)	65.8 (1.0)	
+Vrp	2.6 (25)	1.58 (42)	
Other peptide ionophores			
Valinomycin	0.006 (1.0)	0.04 (6.7)	
+Vrp	0.003 (2.0)	0.00015 (270)	
GmS	15 (1.0)	15 (1.0)	
+Vrp	15 (1.0)	15 (1.0)	
Alamethicin	15 (1.0)	15 (1.0)	
+Vrp	< 2 (> 7.5)	6 (2.5)	
Melittin	17 (1.0)	17 (1.0)	
+Vrp	17 (1.0)	17 (1.0)	
Non-peptide ionophores			
Amphotericin B	70 (1.0)	40 (0.6)	
+Vrp	43 (1.6)	27 (1.5)	
Nigericin	5.5 (1.0)	5.5 (1.0)	
+Vrp	5.5 (1.0)	5.5 (1.0)	
Nonactin	0.54 (1.0)	1.2 (2.2)	
+Vrp	0.33 (1.6)	0.07 (17)	
Monensin	1.5 (1.0)	3.0 (2.0)	
+Vrp	0.75 (2.0)	0.75 (4.0)	

[a] IC_{50} values were interpolated from cytotoxicity curves generated for each compound.
[b] Numbers given in parentheses for each compound indicate cross-resistance relative to the AuxB1 parent.
[c] Numbers given in parentheses for each compound tested in combination with verapamil (Vrp) indicate the chemosensitization index, i.e., the ratio of IC_{50} in the presence of 20 μM verapamil to that in its absence. Verapamil showed no significant toxicity to any of the cell lines tested at this concentration.
All experiments were carried out at least twice, and representative data sets are shown.

Vinca alkaloids and anthracyclines. It is interesting to note that CH^RC5 cells selected for resistance to colchicine were, in our hands, much more cross-resistant to colchicine, vinblastine, adriamycin, and daunomycin (190-, 38-, 40-, and 43-fold, respectively) than were the *mdr1*-transfected LR73/1A cell line (28-, 19, 15- and 15-fold, respectively). Yet the relative resistance of LR73/1A to GmD is 3-fold higher than that of CH^RC5, primarily because of the higher sensitivity of the LR73 parent to GmD toxicity (Tables 1 and 2). The IC_{50} for GmD in the transfected line is similar to that determined for CH^RC5. It seemed possible that overexpression of a large, hydrophobic

integral protein might result in GmD resistance via a nonspecific effect on membrane structure. However, an LR73 transfectant expressing a non-functional mutant *mdr1* protein containing site-directed mutations in highly conserved lysine residues in both of its ATP-binding domains (LR73/88-8) was not resistant to GmD (Table 2). The expression level of mutant Pgp in this cell line is comparable to CH^RC5 (Yu, X. and Sharom, F.J., unpublished data), which indicates that overexpression of a nonfunctional Pgp is not sufficient to confer GmD resistance. Taken together, these data suggest that GmD may be a 'transport substrate' for Pgp.

3.2. Chemosensitization of GmD resistance

GmD toxicity to MDR cell lines was greatly increased by the presence of 20 μM verapamil, a recognized chemosensitizer (Table 1). Verapamil had the effect of moving the IC_{50} for GmD in both AuxB1 and CH^RC5 to lower values (Fig. 1A), but this decrease, as represented by the chemosensitization index (Table 1), was much larger for CH^RC5. These results suggest that verapamil is either directly competing with GmD for a drug-binding site on Pgp [41], or is interacting with Pgp at an allosteric site, to reduce ionophore binding at another site [47]. To investigate the possibility that reversal of GmD resistance by verapamil is a nonspecific phenomenon, and not a specific chemosensitization effect, we tested several known chemosensitizers of MDR [36] for their ability to reverse GmD toxicity. Concentrations of each chemosensitizer were selected to be half the IC_{50}, as determined by the MTT cytotoxicity assay (data not shown). We found that reserpine and quinidine, along with verapamil, were the most potent chemosensitizers. Tween 80 also produced substantial sensitization of GmD toxicity. Quinine produced modest levels of chemosensitization. Trifluoperazine, dipyridamole, and nifedipine had small effects, which were similar in both drug-sensitive and -resistant lines (Table 3). The local anesthetics tetracaine, xylocaine and procaine had little effect, despite reports that tetracaine inhibited the rate of GmD channel formation in planar lipid bilayers [48].

Table 2
Resistance of parental and murine *mdr1*-transfected CHO cell lines to drugs and peptide ionophores

Compound	IC_{50} (μM) [a]		
	LR73	LR73/1A [b]	LR73/88-8 [c]
Colchicine	0.045 (1.0) [d]	1.28 (28)	0.045 (1.0)
+Vrp	0.013 (3.5) [e]	0.038 (34)	0.020 (2.3)
GmD and derivatives			
GmD	0.21 (1.0)	18.4 (88)	0.21 (1.0)
+Vrp	0.026 (8.1)	0.10 (180)	0.026 (8.1)
DeformylGmD	18.9 (1.0)	40.8 (2.2)	
+Vrp	0.81 (23)	2.16 (19)	
Other peptide ionophores			
Valinomycin	0.01 (1.0)	0.07 (7.0)	
+Vrp	0.0004 (25)	0.0007 (100)	

[a] IC_{50} values were interpolated from cytotoxicity curves generated for each compound.
[b] Transfectants expressing the mouse *mdr1* gene product [33].
[c] Transfectants expressing a mutant form of the mouse *mdr1* gene product with site-directed Lys → Arg substitutions in both the N-terminal and C-terminal nucleotide-binding folds [33].
[d] Numbers given in parentheses for each compound indicate cross-resistance relative to the LR73 parent.
[e] Numbers given in parentheses for each compound tested in combination with verapamil (Vrp) indicate the chemosensitization index, i.e., the ratio of the IC_{50} value in the presence of 20 μM verapamil to that in its absence. Verapamil showed no significant toxicity to any of the cell lines tested at this concentration.
All experiments were carried out at least twice, and representative data sets are shown.

Table 3
Chemosensitization of GmD cytotoxicity by various compounds

Compound	Concn. (μM) [b]	IC_{50} (μM) [a]	
		AuxB1	CH^RC5
GmD		0.53	15.8
Calcium channel/calmodulin antagonists			
Verapamil	20	0.095 (5.6) [c]	0.16 (99) [c]
Nifedipine	60	0.13 (4.1)	3.4 (4.7)
Trifluoperazine	12	0.32 (1.7)	6.3 (2.5)
Anesthetics			
Xylocaine	275	0.12 (4.3)	12 (1.3)
Tetracaine	30	0.12 (4.3)	3.2 (5.0)
Procaine	350	0.12 (4.3)	9.9 (1.6)
Amphiphiles			
Tween 80	500	0.03 (17)	0.4 (40)
Triton X-100	8	0.12 (4.3)	4.5 (3.5)
Other compounds			
Reserpine	8	0.09 (5.9)	0.11 (140)
Quinidine	60	0.39 (1.4)	0.21 (75)
Quinine	60	0.21 (2.5)	1.6 (9.9)
Dipyridamole	10	0.13 (4.1)	4.2 (3.8)
Progesterone	15	0.40 (1.3)	15.8 (1.0)

[a] IC_{50} values were interpolated from cytotoxicity curves generated for GmD in the presence of the stated concentration of chemosensitizer.
[b] Verapamil was used at a concentration of 20 μM, while the concentrations of other chemosensitizer were chosen to be half the IC_{50} value. Toxicity towards AuxB1 and CH^RC5 at the stated concentrations was < 10% for all compounds tested.
[c] Numbers given in parentheses for each chemosensitizer tested in combination with GmD indicate the chemosensitization index, i.e., the ratio of the IC_{50} value in the presence of the compound to that in its absence.
All experiments were carried out at least twice, and representative data sets are shown.

3.3. Resistance of MDR cells to deformylGmD

Subtle changes in the chemical structure of GmD can dramatically alter the single channel conductance properties of the peptide. To determine whether GmD was cytotoxic by forming cation-specific channels in the plasma membrane of the cell, we tested MDR cells for resistance to deformylated GmD. Removal of the N-terminal formyl group of GmD is known to prevent peptide dimerization, and this derivative shows very low cation conductance [44]. Table 1 shows that deformylGmD was approximately 125-fold less toxic than GmD to the AuxB1 parent, and the CH^RC5 cell line did not display cross-resistance to this derivative. Similar results were noted for the *mdr1* transfectants (Table 2). Thus the loss of resistance is coincident with the impaired ability of this derivative to form N,N-helical dimers in the bilayer. These results suggest that GmD is cytotoxic to CHO cells via a mechanism related to its ability to form membrane channels. Measurements of Rb^+ efflux further supported this conclusion (see below).

3.4. Resistance of MDR cells to other ionophores

We surveyed the toxicity of a series of ionophores towards MDR CHO cells, to determine whether resistance to GmD was unique, or was perhaps part of a general pattern of cross-resistance to ionophoric agents. The data presented in Table 1 show that CH^RC5

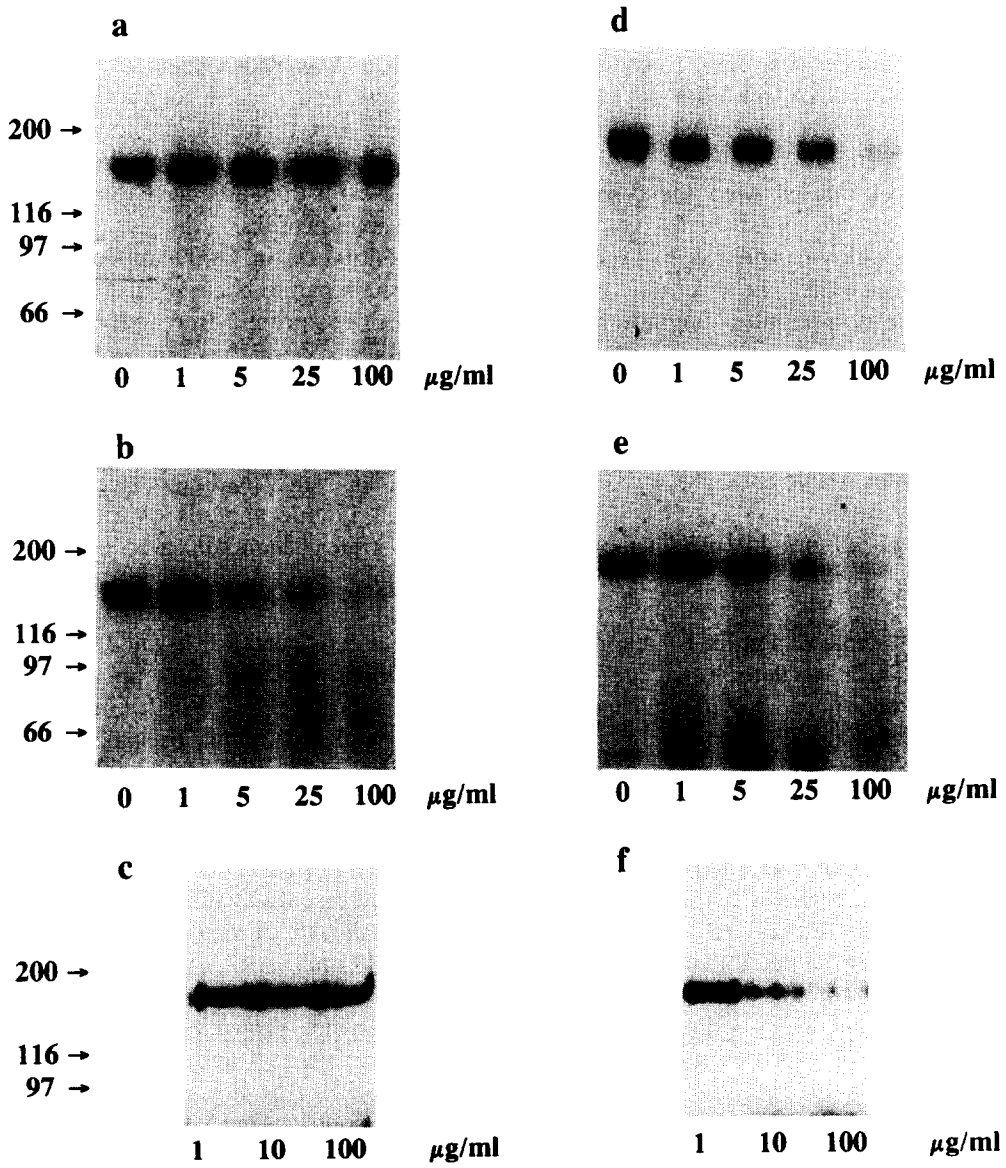

Fig. 2. Inhibition of [^{3}H]azidopine photoaffinity labelling of CH^RC5 plasma membrane vesicles by various ionophores: (a) GmD; (b) GmS; (c) amphotericin B; (d) melittin; (e) valinomycin; (f) alamethicin. Ionophore concentrations are indicated along the bottom of each gel. Arrows and numbers to the left of the gels indicate the position of molecular mass markers in kDa.

displays 6- to 8-fold resistance to valinomycin, a mobile K^+/Rb^+ ionophore that acts by diffusing across the bilayer carrying an ion rather than forming a membrane-spanning pore. *mdr1* transfectants also displayed a similar level of cross-resistance to valinomycin (Table 2). These results are consistent with a previous report that Pgp confers valinomycin resistance in yeast [49]. The chemosensitization index for this compound by verapamil in the drug-resistant lines was also very high, confirming that it is likely a Pgp substrate. MDR cells showed a modest level of cross-resistance to nonactin, a K^+-specific ionophore, which was also sensitized by verapamil (Table 1). In addition, we observed a low level of cross-resistance to monensin, a Na^+-H^+ electroneutral exchanger (Table 1). Magainins, a recently characterized class of peptides from *Xenopus laevis* skin, also appear to function as ion channels in planar lipid bilayers [50]. However, neither magainin I, magainin II, or magainin II amide were toxic to the parent or MDR cell lines, up to 10 μg/ml (data not shown). MDR CHO cells also showed no resistance to the peptide ionophore alamethicin relative to the AuxB1 parent, although both cell lines showed added sensitivity to the compound in the presence of verapamil (Table 1). CH^RC5 cells did not exhibit cross-resistance to any other ion carrier, either the peptides melittin and alamethicin, or the non-peptides amphotericin B and nigericin, nor were they sensitized to these compounds by the presence of verapamil (Table 1). These results indicate that GmD is unique among the ionophores in terms of the high levels of cross-resistance towards it shown by MDR cells.

3.5. Ability of ionophores to block [^{3}H]azidopine photoaffinity labelling of Pgp

In order to assess the role of Pgp in the expression of cross-resistance to GmD, we determined the effects of GmD and other ionophores on photoaffinity labelling of Pgp by [^{3}H]azidopine. Four of the ionophores tested, i.e., GmS, melittin, valinomycin and alamethicin, inhibited photolabelling in a dose-dependent fashion (Figs. 2b, d, e, and f). Inhibition of labelling by melittin and valinomycin required relatively high concentrations, with an IC_{50} between 5 and 25 μg/ml, and complete inhibition at 100 μg/ml. In contrast, 5 μg/ml GmS and 10 μg/ml alamethicin reduced Pgp labelling to less than 20% of the control. These concentrations are near the IC_{50} values for these ionophores. Amphotericin B and (paradoxically) GmD (both at 100 μg/ml) were the only ionophores tested that did not inhibit azidopine labelling of Pgp (Figs. 2a and c). Extending the incubation time to 6 h, or adding 1% bovine serum albumin to facilitate enhanced solubilization of GmD, had no effect on Pgp photolabelling. These results suggest that caution should be exercised when interpreting Pgp azidopine photoaffinity labelling inhibition studies. Physicochemical properties of the plasma membrane, and characteristics of these compounds other than their ability to act as Pgp substrates, may determine the efficacy of photolabelling.

3.6. Uptake of GmD by intact MDR cells

MDR cells may be resistant to GmD because they take up less of the ionophore into the plasma membrane. We initially used the MTT assay to determine the time-frame over which GmD uptake might be observed. We assessed the GmD exposure time necessary to give levels of growth inhibition in AuxB1 that approached those observed for continuous GmD exposure. Twelve different exposure times (1–72 h) were tested (data not shown). High concentrations of GmD (25–50 μM) were cytotoxic after 1 h, while concentrations less than 5 μM required at least 6 h to cause levels of growth inhibition comparable to those seen for continuous exposure to the ionophore. Exposure times greater than 20 h gave essentially superimposable cytotoxicity profiles.

Quantitation of GmD uptake proved to be technically challenging, because of the low solubility of the ionophore in protein-free aqueous buffers [51]. Uptake experiments were thus conducted in culture medium, since this is the vehicle in which toxicity was monitored and cross-resistance observed. However, the use of a complex medium complicates the interpretation of the molecular events leading to uptake, and precludes any rigorous kinetic analysis of the data. Uptake experiments were conducted over a period of 6 h, using ^{125}I-GmD concentrations in the range 0.5–5 μM. Around 5% of the labelled peptide became associated with the cells over 4–6 h, under these conditions. Both AuxB1 and CH^RC5 took up approximately $4 \cdot 10^{-17}$ mol GmD per cell ($2.5 \cdot 10^7$ GmD monomers per cell) when incubated in the presence of 5 μM GmD over this time period (Fig. 3A). GmD uptake increased rapidly over the first 60 min, started levelling off after 3 h, and did not increase significantly at times longer than 6 h. Both CH^RC5 and the drug-sensitive parent displayed essentially identical uptake of ^{125}I-GmD over the 6 h period. A similar profile was observed for 0.5 μM ^{125}I-GmD, with the level of uptake being about $5 \cdot 10^{-18}$ mol GmD per cell, or $3 \cdot 10^6$ monomers per cell (data not shown). This concentration is of some relevance, since it approximates the IC_{50} for AuxB1, and suggests that these cells can tolerate the adsorption of $3 \cdot 10^6$ GmD molecules to the cell surface before the ionophore exerts its toxic effects. This value is 30-fold greater than the amount of cell-associated GmD that was reported to be necessary to induce detectable changes in the flip-flop rate of lysophospha-

tidylcholine in the erythrocyte membrane [52]. The difference in total cellular uptake in CHO cells and erythrocytes required to produce an observable effect may arise from the fact that two different processes are being measured (cytotoxicity vs. flip-flop), as well as from dissimilarities in size and surface area between cell types. ^{125}I-GmD uptake over a 40-min period increased linearly up to a concentration of 2 μM, with only a slight levelling off in uptake noted at higher concentrations. Again, both MDR and drug-sensitive cell lines showed identical concentration dependence of GmD uptake, which suggests that reduced uptake of the ionophore is not responsible for cross-resistance.

3.7. $^{86}Rb^+$ uptake by MDR CHO cells

$^{86}Rb^+$ is often used as a convenient radioactive analogue for Na^+, K^+ and other cations that pass

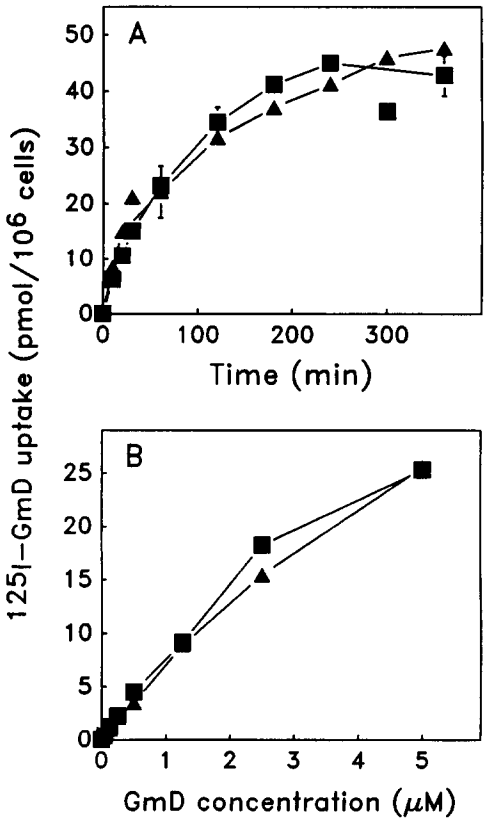

Fig. 3. Time and concentration dependence of uptake of GmD by multidrug resistant CHO cells. (A) After incubation with 5 μM ^{125}I-labelled GmD for various times at 37°C, AuxB1 (▲) and CH^{R}C5 (■) cells with associated GmD were separated from free ionophore by rapid centrifugation (60 s) through an ice-cold cushion of 1 M sucrose (see Materials and methods). Tubes were rapidly frozen in liquid N_2, and the tips containing the cell pellet were sliced off and counted. (B) AuxB1 (▲) and CH^{R}C5 (■) cells were incubated for 40 min at 37°C in medium containing various concentrations of ^{125}I-labelled GmD and processed as above. Background values for controls containing ^{125}I-GmD but no cells were subtracted from experimental data, which are presented as means ± range ($n = 2$).

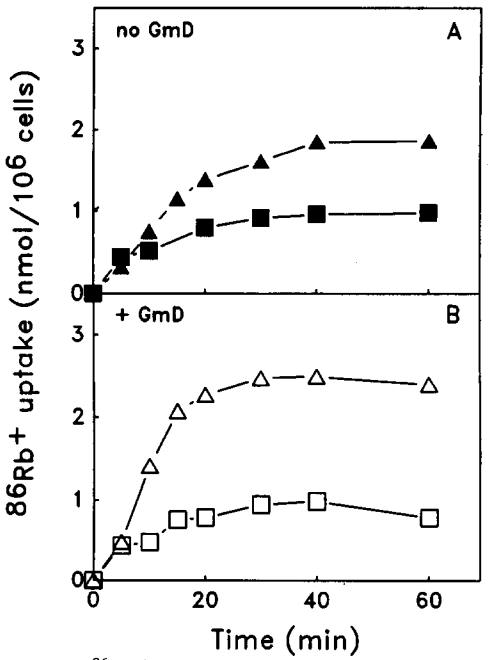

Fig. 4. Uptake of $^{86}Rb^+$ by multidrug resistant CHO cells. AuxB1 (▲,△) or CH^{R}C5 (■,□) cells were incubated in the presence of 1 μCi/ml $^{86}Rb^+$ (2–40 nmol/assay) for various times, in the absence (A) or presence (B) of 5 μM GmD. Cell-associated $^{86}Rb^+$ was determined using the same methods as for Rb^+ efflux. Data are presented as means ($n = 2$). Limits of error (range) fall within the symbols.

through GmD channels. We first noted reduced levels of $^{86}Rb^+$ uptake by CH^{R}C5 cells on pre-loading cells for cation efflux studies (see below). Steady-state levels of $^{86}Rb^+$ accumulated after 60 min were consistently 2-fold lower in CH^{R}C5 cells relative to AuxB1 (Fig. 4A). Levels of uptake for CH^{R}A3 and CH^{R}B30 were similar to AuxB1 and CH^{R}C5, respectively (data not shown). Treatment with 5 μM GmD enhanced the rate of $^{86}Rb^+$ uptake by AuxB1 cells, but had no effect on CH^{R}C5 cells (Fig. 4B). GmD also increased the steady-state levels of $^{86}Rb^+$ accumulation by approximately 30% in AuxB1, but again had no effect on CH^{R}C5. Thus GmD gives rise to enhanced cation uptake in drug-sensitive cells, but not in their drug-resistant counterparts, suggesting that the ionophore is unable to form functional channels in the MDR cell membrane. Differential uptake of $^{86}Rb^+$ was not mirrored by a differential sensitivity of the two cell lines to ouabain or furosemide (data not shown), as previously reported in MDR P388 cells [53]. Rb^+ uptake by AuxB1 and CH^{R}C5 was also affected similarly by procaine, tetracaine, quinidine, and tetraethylammonium (data not shown). These anaesthetics and channel blockers were able to reduce $^{86}Rb^+$ uptake only at concentrations close to their IC_{50}, and at which they typically inhibit membrane-bound cation channels [54].

3.8. $^{86}Rb^+$ efflux from MDR CHO cells

Riordan and Ling reported that the rate of K^+ efflux, in the presence of 1 μM GmD, was elevated in AuxB1 but not CH^RC5 cells [55]. We have extended this observation by measuring $^{86}Rb^+$ efflux from several CHO cell lines with various levels of MDR and Pgp expression. In addition, the GmD- and verapamil-dependence of this efflux was investigated. The effects of GmD on cation efflux were quantitated by calculating the time required for 50% of the cellular Rb^+ to leave the cell ($t_{1/2}$), which was determined by fitting the efflux profile to a single-order exponential decay (Table 4). In the absence of ionophore, AuxB1 and CH^RC5 displayed very similar $^{86}Rb^+$ efflux profiles (Fig. 5A), and the half-time $t_{1/2}$ for efflux was comparable for each (Table 4). Addition of GmD greatly enhanced the rate of $^{86}Rb^+$ efflux from drug-sensitive AuxB1 cells (Fig. 5B), in a concentration-dependent manner (Table 4). At the highest concentration of GmD tested (100 μM), the $t_{1/2}$ was reduced to less than 5% of that observed in its absence. In contrast, the profile of cation efflux from CH^RC5 was only affected slightly by GmD (Fig. 5B), and the $t_{1/2}$ at the highest GmD concentration was reduced by only 35% compared to the untreated control (Table 4). In the presence of 20 μM verapamil, the rate of GmD-dependent Rb^+ efflux from CH^RC5 cells was greatly enhanced, and approached that measured in AuxB1 cells (Fig. 5D), so that the $t_{1/2}$ for efflux from the MDR cell line was comparable with that measured for the sensitive parent (Table 4). Verapamil alone had essentially no effect on Rb^+ efflux from either AuxB1 or CH^RC5 (Fig. 5C). Similar $^{86}Rb^+$ efflux experiments were carried out in the presence of vinblastine, which is a high-affinity substrate for Pgp [8]. As indicated in Table 4, this drug did not affect cation efflux when added alone to either of the cell lines. It also had no effect on GmD-mediated Rb^+ efflux from AuxB1, and produced only a small increase in the GmD-mediated efflux rate in CH^RC5 (Table 4). These results suggest that, unlike verapamil, vinblastine is not able to interact either directly, or indirectly, with the GmD binding site on Pgp.

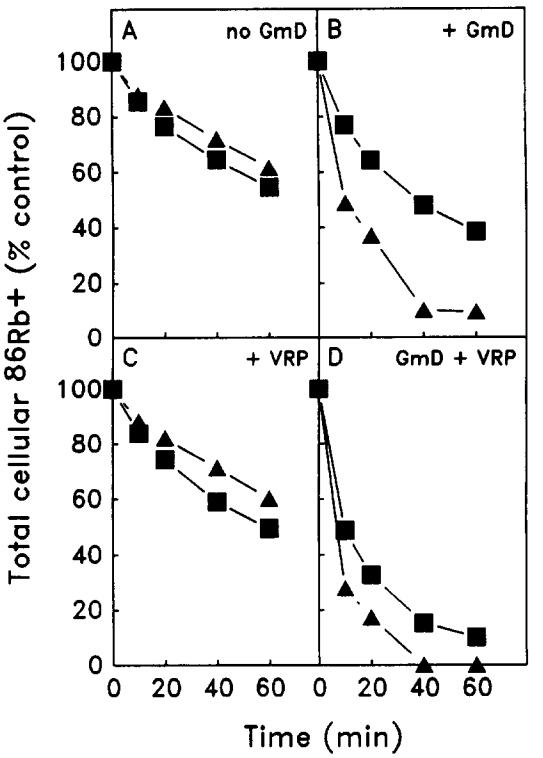

Fig. 5. GmD-enhanced $^{86}Rb^+$ efflux from multidrug-resistant CHO cells. AuxB1 (▲) or CH^RC5 (■) cells were loaded with $^{86}Rb^+$ at 1 μCi/ml for 2 h at 37°C, rapidly washed at 4°C, and then monitored for efflux of radiolabel at 37°C, with no additions (A), or in the presence of either 5 μM GmD (B), 20 μM verapamil (C), or 5 μM GmD together with 20 μM verapamil. Cell-associated $^{86}Rb^+$ was separated from free Rb^+ by rapid centrifugation (60 s) through an ice-cold 1 M sucrose cushion. Tubes were rapidly frozen in liquid N_2, and the tips containing the cell pellet were sliced off and counted. $^{86}Rb^+$ remaining in the cells at various times is shown relative to that at time zero. Data are presented as means ($n = 2$). Limits of error (range) fall within the symbols.

Table 4
Half-times for GmD-mediated $^{86}Rb^+$ efflux from MDR CHO cells

	$t_{1/2}$ (min)	
	AuxB1	CH^RC5
GmD concn. (μM)		
0	65	67
0.5	–	64
2.5	33	55
12.5	14	44
50	3	–
Treatment		
None	86	65
5 μM GmD	12	38
20 μM verapamil	82	55
5 μM GmD + 20 μM verapamil	6	12
None	71	73
5 μM GmD	11	51
5 μM vinblastine	64	66
5 μM GmD + 5 μM vinblastine	9	40

Values for the half-time of Rb^+ efflux ($t_{1/2}$) were calculated by fitting efflux results (e.g., Fig. 5) to a single-exponential decay, which was found to give the best fit to the data. t (min) was solved for $c/c_0 = 0.5$ in the equation $c/c_0 = A \cdot \exp(-kt)$, where A is a constant. Representative data sets are shown, each originating from a separate group of experiments conducted using a different preparation of intact MDR cells.

It is interesting to note that while the levels of cell-associated GmD increased over a 3–4 h period, GmD had a substantial effect on cation efflux within minutes of addition to $^{86}Rb^+$-loaded cells. Indeed, we have determined that, in our experiments, MDR CHO cells take up $(1–12) \cdot 10^8$ Rb^+ ions/cell, depending on

the amount of $^{86}Rb^+$ required to give 1 μCi/ml (20–400 μM). $^{86}Rb^+$ is diluted within the cytoplasmic pool of K^+, which would be approximately 0.15 M, or $1.6 \cdot 10^{11}$ K^+ ions/cell (cellular diameter was estimated to be 15 μm). If we assume that each GmD channel allows the passage of 10^7 ions/s [54], only approximately 53 GmD channels would be required for efflux of the total cellular pool of cytoplasmic Rb^+ in 5 min, a rate which is achievable for AuxB1 treated with 25 μM GmD.

To further characterize GmD-mediated cation efflux, cellular levels of $^{86}Rb^+$ in AuxB1 and CH^RC5 were measured after treatment for 40 min with various concentrations of GmD. As shown in Fig. 6, the concentration-dependence of cation efflux in the MDR cells was very different from that of the drug-sensitive parent. Virtually no Rb^+ was retained in AuxB1 cells at GmD concentrations greater than 2 μM. In contrast, CH^RC5 resisted the cation efflux effects of GmD, and over half the intracellular Rb^+ was retained at GmD concentrations as high as 20 μM. There was a striking resemblance between the GmD dependence of Rb^+ efflux in AuxB1 and CH^RC5, and the cytotoxicity profile of the ionophore towards the two cell lines shown in Fig. 1A. The close correlation between Rb^+ efflux and cytotoxicity implies that the properties of GmD as an ionophore are responsible for its cytotoxic action in CHO cells. Pgp overexpression in MDR cells results in a reduction in cation efflux via the peptide, and leads to high levels of cross-resistance.

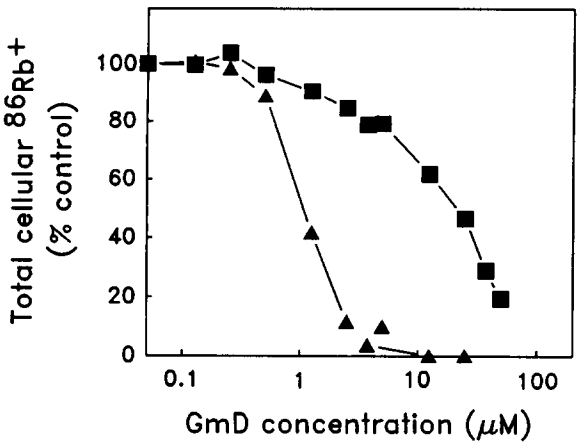

Fig. 6. Effect of GmD concentration on $^{86}Rb^+$ efflux from multidrug-resistant CHO cells. AuxB1 (▲) or CH^RC5 (■) cells were preloaded with $^{86}Rb^+$ and efflux was allowed to take place for 40 min at 37°C in the presence of various concentrations of GmD. Cell-associated $^{86}Rb^+$ was separated from free Rb^+ by rapid centrifugation (60 s) through an ice-cold cushion of 1 M sucrose. Tubes were rapidly frozen in liquid N_2, and the tips containing the cell pellet were sliced off and counted. $^{86}Rb^+$ remaining in the cells is shown relative to that at time zero. Data are presented as means ($n = 2$). Limits of error (range) fall within the symbols.

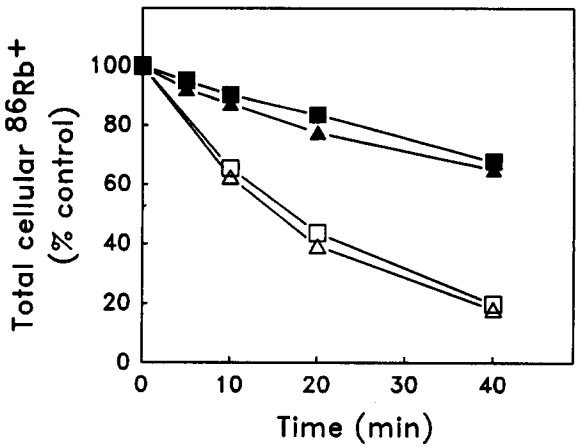

Fig. 7. Valinomycin-dependent $^{86}Rb^+$ efflux from multidrug-resistant CHO cells. AuxB1 (▲,△) or CH^RC5 (■,□) cells were preloaded with $^{86}Rb^+$ for 2 h, rapidly washed, and then monitored for efflux of radiolabel at 37°C in the presence of 1 μM (▲,■) or 100 μM (△,□) valinomycin. Cell-associated $^{86}Rb^+$ was separated from free Rb^+ by rapid centrifugation (60 s) through an ice-cold cushion of 1 M sucrose. Tubes were rapidly frozen in liquid N_2, and the tips containing the cell pellet were sliced off and counted. $^{86}Rb^+$ remaining in the cells is shown relative to that at time zero. Data are presented as means ($n = 2$). Limits of error (range) fall within the symbols.

To determine whether this reduction in cation efflux in MDR cells is unique to GmD, we measured $^{86}Rb^+$ efflux in CHO cells exposed to valinomycin, a mobile ionophore which is also specific for monovalent cations. There was no significant difference in $^{86}Rb^+$ efflux from AuxB1 or CH^RC5 in the presence of either 1 μM or 100 μM valinomycin, despite the cross-resistance of CH^RC5 to this ionophore (Fig. 7). It should be noted that a high valinomycin concentration (around 10^4-fold greater than the IC_{50} for either cell line; see Table 1) was necessary to observe $^{86}Rb^+$ efflux rates above background, in line with the greatly reduced rates of ion transport exhibited by mobile carriers relative to channels. The dissimilar effect of Pgp overexpression on cation efflux via GmD and valinomycin suggests that they may interact with the efflux pump in different ways.

4. Discussion

Certain strains of *Bacillus brevis* synthesize GmD, which is a mixture of three linear 15-amino acid peptides, each consisting of alternating hydrophobic D- and L-amino acids, and modified by an N-terminal formyl group and a C-terminal ethanolamine residue. Gm peptides within this closely related family possess conservative single amino acid substitutions at positions 1 (Val/Ile) and 11 (Trp/Phe/Tyr; GmA, GmB and GmC, respectively). Gm peptides form helical

channels with a central aqueous pore, the linear dimensions of which are sufficient to span half of the lipid bilayer. GmA, GmB and GmC have all been shown to form structurally equivalent ion channels with very similar ion-conducting properties [58]. The mechanism by which GmD forms channels has been studied by ^{19}F- and ^{13}C-NMR and been shown to involve the formation of N,N-helical dimers [59]. GmD is an interesting compound to study within the context of MDR because its channel-forming capability in lipid bilayers is well-characterized, and the topography and orientation of GmD channels is established. In contrast, other drugs within the MDR spectrum have multiple sites of action, and little is known about their disposition within the bilayer [60]. We have established that MDR CHO cells and *mdr1*-expressing transfectants exhibit high levels of cross-resistance to this ionophore; similar results have also been reported for other Pgp-expressing cell lines [21,61–63]. That Pgp is directly involved in mediating GmD resistance is supported by the very high level of cross-resistance seen in *mdr1*-transfectants (higher than several 'drug' substrates), and by the observed lack of resistance to GmD displayed by transfectants expressing a non-functional mutant Pgp (LR73/88-8).

An active role for Pgp in conferring GmD resistance is also implied by the ability of established Pgp chemosensitizers to reverse this resistance. CH^RC5 cells have a very large chemosensitization index for reserpine, verapamil, quinidine, and Tween 80, while less pronounced sensitization was observed for quinine. All of these species are known chemosensitizers for Pgp-mediated multidrug resistance. Since many established chemosensitizers are also ion channel inhibitors (quinine, quinidine, verapamil), and GmD may be toxic to cells by perturbing $\Delta\psi$, it is possible that some of these compounds block the ability of the cell to regenerate $\Delta\psi$. However, given the very specific pattern of chemosensitization noted in Table 3, this seems unlikely. It has been reported that nonionic and zwitterionic detergents modulate GmD channel appearance rate, duration, and conductance [64]. These factors may play a role in the ability of amphiphiles to sensitize CHO cells to GmD toxicity.

The ability of compounds to inhibit photoaffinity labelling of Pgp by azidopine has generally been accepted as implying that the compound of interest binds to, and perhaps is transported by, Pgp. We [35], and others [40,65], have shown that Pgp substrates such as vinblastine and verapamil are potent inhibitors of photolabelling. However, the data obtained in the present study call into question the above interpretation of azidopine photolabelling. Within the group of ionophores tested, the most potent inhibitors of photolabelling were compounds to which CH^RC5 cells were not cross-resistant, whereas GmD had no effect on photolabelling at concentrations up to 50 μM. An alternative explanation for these data is that many membrane-active agents may block labelling by a mechanism that does not involve direct interaction with Pgp. In this regard, we previously reported that many amphiphiles inhibit photolabelling of Pgp at concentrations at which membrane disruption is likely [35]. In addition, the nonionic amphiphiles Triton X-100 and Nonidet P40 disrupted Pgp photolabelling over the same concentration range as vinblastine [35].

The molecular mechanism of toxicity of GmD to eukaryotic cells has not been clearly defined. Studies of cation flux (in either direction) through GmD channels in intact cells are conveniently carried out using the radionuclide $^{86}Rb^+$. Since the dose-dependence of GmD cytotoxicity to CHO cells (Fig. 1A) closely mirrors that observed for Rb^+ efflux (Fig. 6), it appears likely that GmD exerts its toxic effects by acting as an ionophore for monovalent cations at the level of the plasma membrane. However, GmD also modulates transbilayer reorientation of lipids in erythrocyte membranes [42], and induces the formation of hexagonal phase lipid in bilayers [66], and both of these effects could also contribute to GmD-mediated cation efflux.

Time-dependent cytotoxicity assays indicated that low concentrations of GmD (<5 μM) act on CHO cells over a time-frame of about 6 h, and uptake studies using ^{125}I-GmD indicated a non-saturable interaction (up to 5 μM) of the ionophore with the membrane surface, with maximal association occurring after about 5 h. The critical feature of GmD toxicity could be the rate of dimerization within the membrane, rather than the amount of ionophore partitioning into the plasma membrane. At the moment, the mechanism by which exogenously-added GmD redistributes into the inner leaflet of the plasma membrane of intact cells is not clear. The lack of differential uptake of the ionophore between AuxB1 and CH^RC5 cells suggests that either GmD is not translocated into the inner membrane leaflet in resistant cells, or it is redistributed within the bilayer in MDR cells so that its ability to form functional channels is impeded, while its total concentration within the bilayer remains unaltered.

Only a short GmD exposure is required to enhance $^{86}Rb^+$ flux rates in CH^RC5 cells, whereas a longer exposure (6 h) is necessary for toxicity to be apparent. These two observations can be reconciled by proposing that few GmD channels are required to produce the observed cation efflux rates. Indeed, the amount of GmD that actually partitions into the plasma membrane of CHO cells is small, and there is evidence to suggest that GmD may first adsorb to, and align itself parallel with, the bilayer surface prior to insertion into the hydrophobic region [67]. In addition, these workers showed that GmD-mediated currents in planar lipid

bilayers are very low when GmD is added asymmetrically to one side of a bilayer surface [67], as is the case in our experiments on intact cells.

The mechanism(s) by which MDR CHO cells display cross-resistance to GmD has not previously been addressed. There are a number of possibilities to be considered. First, Pgp may actively expel GmD from the bilayer, resulting in lower cellular uptake of the ionophore, or reduced partitioning of GmD into the plasma membrane may result from physicochemical changes associated with MDR. However, the results of uptake experiments with ^{125}I-GmD clearly showed that CH^RC5 cells take up the same amount of GmD as the drug-sensitive parent, thus making these two possibilities unlikely. In contrast, decreased accumulation of MDR spectrum drugs is the norm in these cell lines; e.g., 10-fold lower cellular uptake of daunomycin was observed in intact CH^RC5, compared to AuxB1 (Doige, C.A. and Sharom, F.J., unpublished data), and much lower colchicine accumulation was also reported [55].

Resistance of MDR cells to GmD might be a non-specific membrane effect caused by overexpression of a highly hydrophobic integral protein; for example, GmD might be sequestered by hydrophobic interactions with the transmembrane helices of Pgp. However, this type of effect does not appear to occur, since LR73/88-8 transfectants expressing similar levels of an non-functional mutant Pgp displayed no resistance to GmD (Table 2).

It is possible that the rate of lateral diffusion of GmD monomers in the plasma membrane is impeded in resistant cells, e.g., by reduced membrane fluidity, so that the rate at which functional dimeric GmD channels are formed is reduced. This possibility seems unlikely, since no differences in bulk membrane fluidity were observed in these cell lines [35,55].

Pgp has been proposed to act as a 'flippase' [22], moving hydrophobic substrates from the inner leaflet to the outer leaflet of the plasma membrane. One appealing hypothesis for the ability of Pgp to interfere with GmD action involves the active 'transport' of GmD from the inner to the outer leaflet. While the total amount of GmD present in the bilayer would remain unchanged, the number of functional dimeric channels able to form would be greatly reduced. This mechanism is consistent with our observations of similar GmD uptake in both resistant and sensitive cells, and greatly reduced GmD-mediated Rb^+ uptake and efflux from MDR cells.

There is some recent indirect evidence which suggests that Pgp may transport peptides. Cells expressing *mdr1* were 50-fold resistant to the tripeptide *N*-acetyl-leucyl-leucyl-norleucinal (ALLN), and this resistance was reversed by verapamil [68]. In addition, the ability of *mdr1* to complement *ste6* mutations [69] indicated that Pgp exports the a-factor mating peptide in yeast. Systematic screening of peptides of variable length and hydrophobicity should be possible, to identify the essential features necessary for binding and/or transport by Pgp.

While the GmD residues critical for channel conductivity have been well-characterized, the residues required for putative interaction with Pgp remain to be elucidated. GmD resembles conventional MDR substrates in that it is hydrophobic, and of relatively low molecular mass. Future work in this area could involve a systematic modification of the amino acids within GmD, and quantification of their effect on cross-resistance, cytotoxicity, and cation efflux.

The ability of Pgp to mediate very high levels of resistance to GmD appears to be unique, in that only low levels of resistance were observed for many other peptide and non-peptide ionophores (Table 1). MDR cells demonstrated significant resistance to valinomycin, but results showed that Pgp did not modify the rate of cation efflux via this mobile carrier. It is perhaps noteworthy that GmD is the only ionophore tested whose toxic action requires the coming together of two separate structures in each leaflet of the bilayer. GmD is unlike most other protein ionophores found in nature, which form channels either by oligomerization within the bilayer (e.g., complement C9 or perforin), or by unfolding at the membrane surface to insert a hydrophobic channel-forming domain into the bilayer (e.g., colicin E1). Further study of the interaction of MDR cells with GmD may shed more light on the role of Pgp in this phenomenon.

5. Acknowledgments

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