Differential effects of mitomycin C and doxorubicin on P-glycoprotein expression

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Previous studies have demonstrated that mitomycin C (MMC) and other DNA cross-linking agents can suppress *MDR1* (multidrug resistance 1) gene expression and subsequent functional P-glycoprotein (Pgp) expression, whereas doxorubicin and other anthracyclines increase *MDR1* gene expression. In the present study, with stably transfected Madin–Darby canine kidney C7 epithelial cells expressing a human Pgp tagged with green fluorescent protein under the proximal human *MDR1* gene promoter, we demonstrated that MMC and doxorubicin have differential effects on Pgp expression and function. Doxorubicin caused a progressive increase in the cell-surface expression of Pgp and function. In contrast, MMC initially increased plasma membrane expression and function at a time when total cellular Pgp was constant and Pgp mRNA expression had been shown to be suppressed. This was followed by a rapid and sustained decrease in cell-surface expression at later times, presumably as a consequence of the initial decrease in mRNA expression. These studies imply that there are at least two independent chemosensitive steps that can alter Pgp biogenesis: one at the level of mRNA transcription and the other at the level of Pgp trafficking. Understanding the combined consequences of these two mechanisms might lead to novel chemotherapeutic approaches to overcoming drug resistance in human cancers by altering either Pgp mRNA expression or trafficking to the membrane.

Key words: ATP-binding cassette protein, multidrug resistance, protein trafficking.

INTRODUCTION

Multidrug resistance (MDR) represents a significant clinical barrier to the successful treatment of many human tumours with chemotherapy. Many neoplasms exhibit an MDR phenotype either before treatment (*de novo*) and/or after treatment (acquired) (reviewed in [1–5]). Many mechanisms for MDR have been described, including the up-regulation of one or more membrane proteins to lower intracellular drug concentrations, changes in Phase I and Phase II metabolic pathways for activation/detoxification of drugs, changes in DNA repair response and changes in apoptotic responses to toxic insult. One of the most common and well-described contributors to the MDR phenotype is the overexpression of P-glycoprotein (Pgp), which is a transmembrane ATP-dependent drug translocation protein [1–5]. The specific biophysical mechanism by which Pgp lowers the intracellular concentrations of drugs remains controversial. However, it is clear that the overexpression of Pgp is associated with a clinical MDR phenotype and a poor prognosis for many human cancers [2,5–9].

Pgp is a member of the ATP-binding cassette (ABC) transmembrane protein family, which includes: the MDR-associated protein ('MRP'), whose overexpression is also associated with multidrug resistance [10]; the cystic fibrosis transmembrane conductance regulator protein (CFTR), mutations of which cause cystic fibrosis [11]; and the sulphonylurea receptor ('SUR') [12]. Pgp is expressed in a variety of normal tissues, including liver, kidney and colon; not surprisingly, tumours arising from these tissues usually overexpress Pgp as part of their MDR phenotype [9,13–15]. However, Pgp can also be overexpressed in tumours from tissues that do not normally express, such as breast [14,16]. The mechanism of Pgp up-regulation in tumours *in vivo* is still unclear, but it can occur *de novo* as in acute myelogenous leukaemia [6,15] or can be acquired over the course of cancer treatment as in breast and ovarian cancer [8,15,16].

Previous work by several laboratories has demonstrated that *MDR1* gene transcription and MDR1 mRNA expression can be induced by certain DNA-damaging agents, including chemotherapeutic drugs such as doxorubicin, simple alkylating agents such as methyl methanesulphonate, and genotoxic chemical carcinogens that induce bulky DNA adducts, such as aflatoxin B_1 and 2-acetylaminofluorene [17–22]. In contrast, our laboratory has recently shown that Pgp mRNA and overall protein expression can be significantly suppressed by treatment with DNA cross-linking agents, including the cancer chemotherapy drugs mitomycin C (MMC), cisplatin, carboplatin and BMS181174, and the carcinogen chromium(VI) [12]. We are currently examining the mechanistic basis for this suppression and its possible utility as part of a combination cancer chemotherapy regimen; however, the principal mechanism for the suppression seems to be a down-regulation of *MDR1* gene transcription occurring immediately after treatment with drug. We have hypothesized that this is primarily a gene-selective nuclear response to the DNA cross-links that are formed by these agents [12,23–25].

However, preliminary results from our laboratory also suggested that immediately after treatment with MMC there was a transient increase in membrane-associated Pgp during a period when there was no net increase in total cellular Pgp and when mRNA levels were actually decreasing. On the basis of these

Abbreviations used: ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; GFP, green fluorescent protein; MDCK, Madin–Darby canine kidney; MDR, multidrug resistance; MMC, mitomycin C; Pgp, P-glycoprotein.

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observations we postulated that there might be a second point of Pgp regulation that mediates changes in Pgp trafficking from an intracellular pool to the cell surface in direct response to a toxic chemical challenge. The purpose of these studies was to test this hypothesis in detail. As one way of examining this, we developed a genetic construct expressing a green fluorescent protein (GFP)-tagged human Pgp protein under the transcriptional control of the human *MDR1* promoter. We used this construct to generate stably transfected cell lines in the Madin–Darby canine kidney (MDCK)-C7 parental line. These cell lines were used to investigate the effects of MMC on the expression of GFP–Pgp. These studies demonstrate that this stably transfected GFP–Pgp is fully functional and is expressed in a similar manner to native Pgp, and that doxorubicin and MMC have differential effects on Pgp expression. Our results suggest that there are at least two independent levels of chemosensitive regulation of Pgp expression. The first has previously been well described and is at the level of MDR1 mRNA transcriptional expression. The second is at a post-transcriptional and post-translational level which probably involves trafficking of functional Pgp to the plasma membrane, resulting in a net movement of Pgp from a cytoplasmic pool to the cell surface in response to treatment with drug.

EXPERIMENTAL

Cell culture and treatments

H4IIE rat hepatoma cells were cultured as described previously [12]. MDCK-C7 cells were a gift from Dr Hans Oberleithner (Wurzburg, Germany). Cells were grown in minimal essential medium (Gibco BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 50 units/ml penicillin (Sigma Chemical Co., St Louis, MO, U.S.A.), 50 μg/ml streptomycin (Sigma) and 2 mM L-glutamine (Gibco BRL). The cells were incubated at 37 °C with air/CO_2 (19:1), provided with fresh medium every 2 days and passaged once a week. Treatments were performed in serum-free medium for 4 h unless otherwise stated. Control cells were treated with solvent alone in serum-free medium. After treatment, completed medium was added back to the cells for time points longer than 4 h.

Cloning

The human MDR promoter (hMDRpro, −996 to +44) [22] was amplified by PCR from the metastatic breast cancer cell line MDA-MB-435 cells (A.T.C.C., Manassas, VA, U.S.A.). Oligonucleotide primers 5′-ACGATTAATTTAAAGAAAGTGGAAACA-3′ [sense strand; bases −996 to −979 (*Ase*I restriction site)] and 5′-CTAGCTAGCCCTAAAGGAAACGAACAG-3′ [anti-sense strand; bases +44 to +61 (*Nhe*I restriction site)] were used to amplify a 1080 bp fragment. Primers were designed with Oligo v4.04 Primer Analysis Software (Plymouth, MN, U.S.A.) and synthesized by the Dartmouth College Molecular Biology Core Facility. PCR reaction mixtures (100 μl) contained 100 ng of MDA-MB-435 genomic DNA, each dNTP at 200 μM, each primer at 200 nM, 1.5 mM $MgCl_2$ and 0.5–1.0 unit of *Taq* polymerase (Perkin Elmer, Foster City, CA, U.S.A.). Hot-start PCR was performed with the following cycling parameters: (1) 94 °C for 1 min, (2) 2 cycles of 1 min at 94 °C, 2 min at 55 °C and 2 min at 72 °C, (3) 24 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, and (4) a final extension at 72 °C for 10 min. Control PCR reactions lacking *Taq* polymerase, DNA template or primers did not yield detectable reaction products. PCR products were electrophoretically separated on a 1% (w/v) low-melting-point agarose gel (Life Technologies, Gaithersburg, MD, U.S.A.) in Tris/acetate/EDTA buffer [40 mM Tris/acetate/2 mM EDTA (pH 8.5)], gel-purified with the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, U.S.A.) and subcloned into the pCR 3.1 vector with the use of the Eukaryotic TA Cloning Kit (Invitrogen, San Diego, CA, U.S.A.). Automated DNA sequence analysis was performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Boston, MA, U.S.A.).

The GFP expression vector pEGFP-C1 (Clontech, Palo Alto, CA, U.S.A.) was digested with *Ase*1 and *Nhe*1; the cytomegalovirus promoter was excised. pCR 3.1/human MDR (hMDR) promoter was also digested with *Ase*1 and *Nhe*1 and the hMDR promoter was ligated into the pEGFP-C1 vector in place of the cytomegalovirus promoter to generate hMDRpro/pEGFP-C1.

The human MDR gene 1 cDNA (hMDR1) was a generous gift from Dr William Skach (Oregon Health Science Center, Portland, OR, U.S.A.), originally from Dr. M. M. Gottesman (National Cancer Institute, Bethesda, MD, U.S.A.). Owing to a limited number of restriction sites, the cDNA for hMDR1 was subcloned in two steps. First, the construct hMDRpro/pEGFP-C1 was digested with *Bgl*II, treated with Klenow enzyme, then digested with *Eco*RI. hMDR1 was digested with *Bst*UI and *Eco*RI, and the resulting 1100 bp piece of the hMDR1 cDNA (*Eco*RI, *Bst*UI) was ligated into the hMDRpro/pEGFP-C1 vector to generate hMDRpro/pEGFP-C1/hMDR1–1100 bp. Secondly, hMDRpro/pEGFP-C1/hMDR1–1100 bp was digested with *Eco*RI/*Sma*I, and MDR1 was digested with *Eco*RI/*Pme*I. The 3300 bp fragment of the hMDR1 cDNA (*Eco*RI, *Pme*I) was ligated to the hMDRpro/pEGFP-C1/hMDR1–1100 bp fragment to yield hMDRpro/pEGFP-C1/hMDR1. All ligation sites were sequenced to verify the correct reading frame.

Stable transfections

MDCK-C7 cells were plated at 600000 cells per T75 flask (Corning, Acton, MA, U.S.A.); 24 h after plating, cells were transfected with MDR-GFP DNA in PFx-2 PerFect lipid (Invitrogen) in Opti-MEM medium (Gibco BRL) without any supplements, in accordance with the manufacturer's protocol, with a lipid-to-cDNA ratio of 3:1. After 8 h the DNA/lipid mixture was removed and replaced with completed medium. At 12 h after transfection, 0.3 mg/ml G418 (Gibco BRL) was added to the medium to select for cells expressing the hMDRpro/pEGFP/hMDR1 construct. At 2 weeks after transfection, GFP-positive cells were sorted into 96-well plates (Falcon; Becton Dickinson, Franklin Lakes, NJ, U.S.A.) at a density of one cell per well by a FACStar Plus cell sorter (Becton Dickinson, San Jose, CA, U.S.A.). Cells were clonally expanded, and three clones designated MDR4, MDR10, MDR29 were selected on the basis of bright green fluorescence as detected by a fluorescence microscope (Axiophot; Zeiss) equipped with a halogen lamp and a FITC filter set. These clones were characterized further in detail.

Western blot analysis

Cells were grown to 70% confluence on six-well plates. After treatment with drug, cells were lysed and total or plasma membrane proteins were collected as follows. For total proteins, cells were lysed in 50 mM Tris/HCl (pH 8.0)/150 mM NaCl/1% (v/v) Nonidet P40 containing a protease inhibitor cocktail (Complete Mini; Roche Diagnostic Corp., Indianapolis, IN, U.S.A.) on ice. Detergent-insoluble materials were removed by centrifugation at 12000 ***g*** for 10 min. Plasma membrane fractions

were purified as described previously [26]. Cells were overlaid for 10 min at room temperature with 20 mM Tris/HCl (pH 7.4)/2 mM EDTA/1 mM EGTA/0.1 % digitonin (Sigma) containing protease inhibitors, as described above. Cells were scraped, homogenized and centrifuged at 12000 ***g*** for 10 min. The supernatant containing the cytosolic fraction was removed from each sample. The crude plasma membrane proteins were solubilized in the Nonidet P40 buffer described above. SDS/PAGE was performed under reducing conditions on 4–15 % (w/v) minigels with 10 μg of protein lysate per lane. Proteins were separated and transferred to PVDF membrane (Millipore, Bedford, MA, U.S.A.) in Towbin's transfer buffer [25 mM Tris/HCl/192 mM glycine/12 % (v/v) methanol]. After transfer, the blots were blocked and probed sequentially, first with C219 (Centocor, Malvern, PA, U.S.A.) or F4 (Neomarkers, San Diego, CA, U.S.A.) overnight at 4 °C and then with a horseradish-peroxidase-labelled anti-mouse IgG secondary antibody (Amersham, Piscataway, NJ, U.S.A.). Membranes were washed six times in PBST [PBS/0.3 % (v/v) Tween 20] at room temperature for 10 min each between probings. The blots were developed with enhanced chemiluminescence substrate (Pierce) and exposed to film. For GFP detection, blots were probed with an anti-GFP antibody (Clontech). Digital densitometry was performed as described previously [12].

Accumulation of daunorubicin

To assess Pgp function, cellular accumulation of the fluorescent Pgp substrate drug, daunorubicin, was assayed by flow cytometry. Cells were seeded at 70 % confluence and treated for 24 h with 20 μM verapamil (Sigma) for 1.5 h to block the transport of daunorubicin. After treatment with verapamil, all flasks were treated with 1 μM daunorubicin (Calbiochem, San Diego, CA, U.S.A.) for 1 h. After the 1 h daunorubicin treatment, the T25 flasks were washed with PBS (without Ca^{2+} or Mg^{2+}) and the cells were trypsinized (Trypsin EDTA; Gibco BRL), removed from the flask, pelleted by centrifugation, resuspended in PBS (containing Ca^{2+} and Mg^{2+}) and immediately analysed by FACScan (Becton Dickinson). For each sample 10000 cells were analysed for GFP (green, excitation at 488 nm, emission at 507 nm) and daunorubicin (red, 488 nm/575 nm) fluorescence. Data analysis was performed with CellQuest (Becton Dickinson) software.

Flow cytometric detection of GFP and Pgp expression

Evaluation of Pgp expression in flow cytometric studies of the MDCK/MDR29 cell line was determined with a high-affinity Pgp monoclonal antibody that recognizes an external epitope of the protein [27] conjugated with phycoerythrin with an F/P ratio of 1.0 (Becton Dickinson). Antigen-saturating protein concentrations of the primary and isotype-matched, PE-conjugated, control antibody (Pharmingen) were incubated for 30 min at 20 °C with 250000 cells from each sample per 50 μl in PBS with 0.1 % sodium azide in accordance with the manufacturer's recommendations. Cells were washed, pelleted, resuspended in 500 μl and fixed with 1 % (w/v) paraformaldehyde and analysed immediately. Ten thousand events were collected per sample in list-mode files during laser excitation in the FACScan Analyzer with LYSIS II software (Becton Dickinson) for each experiment. Identical detection settings were used for all studies. Data were processed with CellQuest software (Becton Dickinson).

All studies were conducted in triplicate with sham-treated controls corresponding to each time point. Time-course evaluations of Pgp and GFP expression were conducted with non-cytotoxic doses of MMC and doxorubicin as defined below. Cells were plated in T25 flasks (Falcon) to ensure 30–70 % confluence during the time-course studies (96 h). Viability in selected samples throughout the time-course studies was determined by the addition of 7-aminoactinomycin D (Viaprobe™; Pharmingen). Pgp and GFP expression in non-viable cell populations were excluded. The median fluorescence of the primary Pgp antibody was corrected for non-specific antibody fluorescence in both the drug-treated and sham-treated samples. Pgp and GFP fluorescence were normalized relative to pre-treatment values.

Cytotoxicity assay

Cells were plated in 96-well plates (Fisher, Medford, MA, U.S.A.) at a density of 1000 cells per well; 24 h after plating, the cells were dosed with 0–10 μM doxorubicin in serum-free medium as described above. The cells were incubated for 3 days and assayed with the CellTiter 96 AQueous One Solution Reagent (Promega) in accordance with the manufacturer's instructions. The data were collected and analysed with Softmax software. A cytotoxicity assay was also performed with cisplatin (0–30 μM) as described above.

Statistics

Statistical analysis was performed with InStat for Macintosh software (version 2.0b; GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Our previous studies on the effects of MMC on Pgp mRNA and protein expression had demonstrated an overall suppression of MDR1 mRNA and total cellular Pgp levels in several rat and human cell lines [12]. However, in the present study we re-examined this phenomenon at a more detailed level initially in the rat hepatoma H4IIE cells (Figure 1), comparing levels of

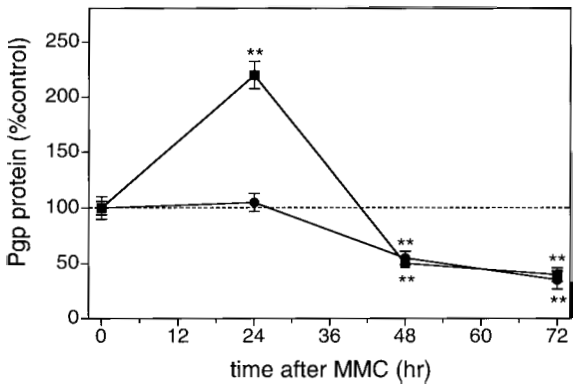

Figure 1 Differential effects of MMC on total cellular and membrane-associated Pgp levels

Rat hepatoma H4IIE cells were cultured and treated with 0.1 μM MMC for 4 h as described previously [12]. Western analysis of Pgp levels was performed with 50 μg per sample of either total cellular protein (●) or membrane protein fraction (■) extracts from treated cells by using the C219 monoclonal antibody as described previously [12] and in the Experimental section. Images were scanned digitally and quantified by digital densitometry as described previously and in the Materials and methods section. Results are expressed as percentages of the control (solvent-treated) value at each time point. There was no significant change in control values over the course of the experiment (results not shown). Results are mean ± S.E.M. for determinations from four to six individual cultures per treatment. Asterisks denote values that were statistically different from control at $P < 0.01$ (Student's t test).

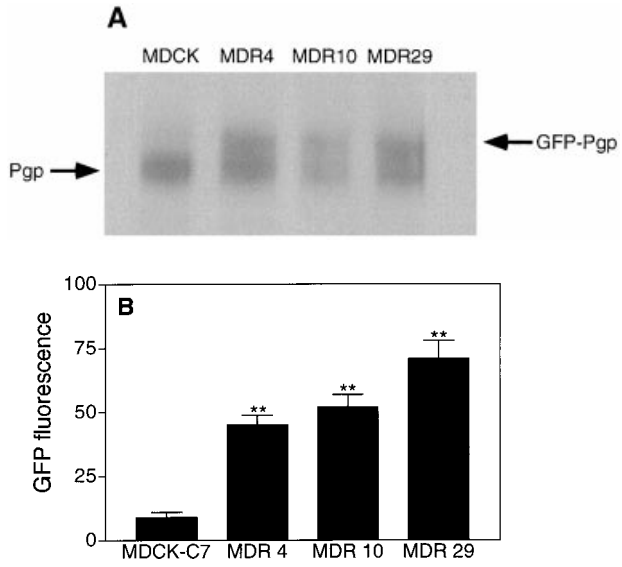

Figure 2 Stable expression of GFP–Pgp fusion protein in independently cloned MDCK sublines

MDCK-C7 cells were stably transfected with a construct containing a GFP-tagged human Pgp fusion protein under the transcriptional control of the human *MDR1* gene promoter. After the selection of independent cell lines by flow cytometric cell sorting for GFP fluorescence, three lines were chosen for further detailed characterization and were designated MDR4, MDR10 and MDR29. (**A**) Western blot analysis of membrane protein fractions from each line was performed with the C219 antibody to detect Pgp as described in the legend to Figure 1. A band corresponding to the native canine Pgp (approx. 180 kDa) was detected in all four cell lines, and a band corresponding to the predicted size of the GFP–Pgp hybrid protein (approx. 210 kDa) was observed in the three transfected cell lines at various levels of expression. (**B**) GFP fluorescence of each cell line was analysed by flow cytometry (relative fluorescence units). Results are means ± S.E.M. for determinations from three individual cultures, where each sample consisted of approx. 10000 individual cells. Asterisks denote values that were significantly different from the parental line ($P < 0.01$, Student's t test).

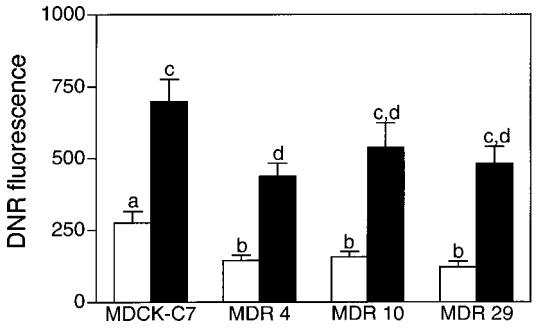

Figure 3 Drug accumulation as a measure of functional membrane Pgp levels in parental and stably transfected MDCK cell lines

Cells were incubated with the fluorescent Pgp substrate drug daunorubicin (DNR), either alone (open bars) or in the presence of the Pgp inhibitor verapamil (filled bars), then analysed by flow cytometry as described in the Experimental section and in the legend to Figure 2. Results are means ± S.E.M. for determinations from three individual cultures per treatment (a minimum of 10000 cells were counted per sample). Bars labelled with different letters denote values that were significantly different from each other ($P < 0.01$, Student's t test).

total and plasma-membrane-associated Pgp. We observed a transient approx. 2-fold increase in membrane-associated Pgp within the first 24 h after treatment with MMC, during a period when total cellular Pgp levels were unchanged (Figure 1). At subsequent time points there was a parallel decrease in both total and membrane Pgp to levels well below control; this seemed to be a consequence of the significant decrease in MDR1 mRNA levels previously observed within the first 24 h after treatment with MMC [12]. This suggested that MMC, and perhaps other drugs, might be simultaneously affecting both *MDR1* gene expression and the cellular localization of Pgp protein.

To assess the effects of drugs on membrane Pgp protein expression more directly, we developed a cDNA construct expressing a GFP-tagged human Pgp protein under the control of the human *MDR1* promoter region (see the Experimental section). This construct was stably transfected into MDCK-C7 cells. Single cells expressing GFP were isolated by FACS, independent cell lines were established by subcloning and these lines were then characterized for GFP–Pgp expression. As shown in Figure 2(A), three different cell lines, designated MDR4, MDR10 and MDR29, expressed a protein that was detectable by Western blotting with an anti-Pgp antibody, which migrated at a position equivalent to the higher predicted size of the hybrid protein (approx. 210 kDa, consisting of 180 kDa Pgp plus 27 kDa GFP) and was not detected in the C7 parental line. An anti-GFP antibody recognized this higher-molecular-mass band in the three transfected cell lines but not in the parental line (Figure 2A), indicating that this corresponded to the full-length hybrid GFP–Pgp protein. Moreover, there was little or no cross-reacting signal at lower molecular masses for either antibody (results not shown). This indicated that alternative forms and/or breakdown products of the hybrid protein were not present to a significant degree and that the predominant expressed form in these cells was that of full-length GFP–Pgp protein. The parental line and the three transfected cell lines also expressed a protein corresponding to the size of native canine Pgp (approx. 180 kDa), which was also detectable with C219 (Figure 2) but not with human-specific antibodies (results not shown). The mean GFP fluorescence of cells from the three MDR cell lines (Figure 2B) was closely correlated with their level of expression of GFP–Pgp as shown by Western blotting (Figure 2A).

Daunorubicin accumulation, as measured by flow cytometry, was then used to assess functional Pgp in each cell line. Daunorubicin is a fluorescent Pgp substrate; cellular accumulation of this drug has been shown to be a sensitive and accurate measure of functional Pgp in cells. As shown in Figure 3, the three MDR cell lines demonstrated significantly less daunorubicin accumulation than did the parental cell line. These results suggested that these cell lines were expressing the GFP–Pgp protein in a functional form on the cell surface, such that their total Pgp levels were greater than that of the parental cell line. In addition, treatment of the cells with the Pgp inhibitor verapamil increased daunorubicin levels in both the parental and MDR cell lines, although drug accumulation in the presence of verapamil was less in the MDR cell lines than in the parental line. However, because verapamil does not fully block Pgp function, this is also consistent with the higher level of functional GFP–Pgp protein on the cell surface of the transfected cells than in the parental cell line.

Cytotoxicity assays also confirmed an increase in functional expression of Pgp in the transfected cells. As shown in Table 1, the EC_{50} for cytotoxicity (colony formation) by the Pgp substrate drug, doxorubicin, was approx. 2.5–3.5-fold greater in the MDR cell lines than in the parental cell line, whereas the EC_{50} values for cytotoxicity by the non-Pgp substrate drug, cisplatin, were identical in all four cell lines. These results are consistent with the conclusion that these three MDR cell lines expressed full-length hybrid GFP–Pgp that was expressed on the cell surface in a form that was functionally similar to that of the native Pgp protein.

Table 1 Relative toxicity of doxorubicin and cisplatin in the parental and stably transfected MDCK cell lines

Cytotoxicity was measured by a colony-forming assay as described in [12] and in the Experimental section. Cytotoxicity dose–response curves were generated for each drug in each cell line by treating triplicate cultures for each concentration (4 h) over a 100-fold range of doses (results not shown); the EC_{50} for cytotoxicity was calculated from each curve by using the standard dose–response curve-fitting equations of Prism statistical software (version 2.0b; Graphpad Software).

Cell line	EC_{50} for doxorubicin (μM)	EC_{50} for cisplatin (μM)
MDCK-C7	1.2	12
MDR4	3.0	12
MDR10	4.0	12
MDR29	4.0	12

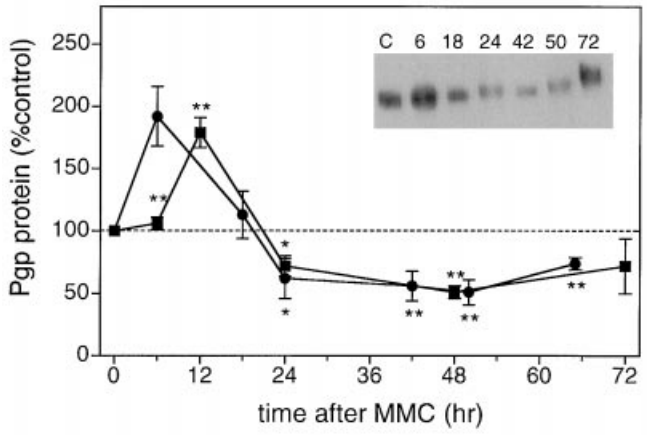

Figure 4 Effects of MMC on plasma-membrane GFP–Pgp expression in MDR29 cells

Cells were treated with 0.1 μM MMC for 4 h and analysed for GFP–Pgp levels. Western blotting of membrane protein samples (10 μg per sample) was performed as described in the Experimental section and in the legends to Figures 1 and 2 above with the use of the F4 monoclonal antibody. A representative blot is shown in the inset (note that there was no change in apparent mobility as determined by standards; the apparent upward shift across this gel was a result of a 'smile'). Densitometric analysis of similar blots for each set of samples was performed as described in [12] and in the Materials and methods section; the results are expressed as percentages of control (solvent) values (●). Results are means ± S.E.M. for values from three individual cultures for each treatment time. Alternatively, after treatment with MMC, intact cells were analysed by flow cytometry with a fluorescently labelled antibody against an external epitope of human Pgp (BD PGP) as described in the Experimental section and in the legends to Figures 2 and 3 above (■). Results are means ± S.E.M. for values from three individual cultures (a minimum of 10000 cells were counted per sample). Asterisks denote values that were significantly different from the corresponding control: $^{*}P < 0.05$, $^{**}P < 0.01$; Student's t test.

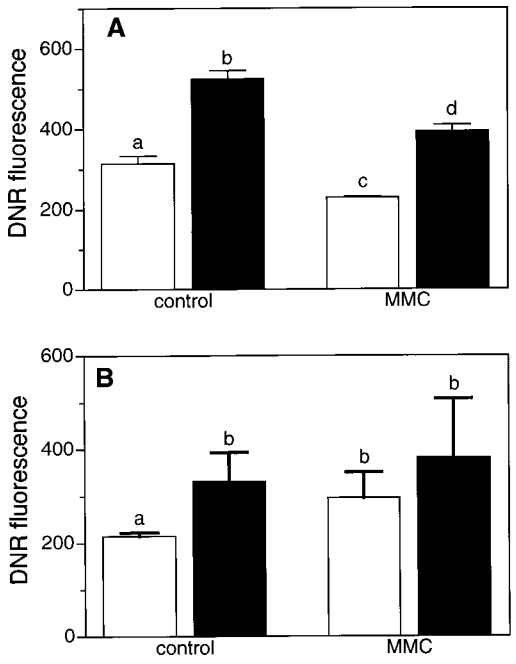

Figure 5 Effects of MMC on drug accumulation in MDR29 cells

Cells were treated with MMC for 12 h (**A**) or 48 h (**B**); daunorubicin (DNR) accumulation was measured in control cells (solvent, 12 or 48 h control) or MMC-treated cells (0.75 μM, 4, 12 or 48 h after treatment) in the absence (open bars) or presence (filled bars) of verapamil by flow cytometry as described in the legend to Figure 4 and in the Experimental section. Results are means ± S.E.M. for determinations from three individual cultures per treatment (a minimum of 10000 cells were counted per sample). Bars labelled with different letters denote values that were significantly different from each other ($P < 0.01$, Student's t test).

Subsequent experiments were performed with the MDR29 cell line, because this was the highest expresser of the GFP–Pgp protein.

MDR29 cells were treated with a single non-cytotoxic dose of MMC; GFP–Pgp protein expression was measured by two different approaches. As shown in Figure 4, MMC treatment caused a doubling of GFP–Pgp levels at 6 h as detected by Western blotting in plasma-membrane-enriched preparations. Measurement of Pgp by flow cytometry with an antibody against an extracellular epitope demonstrated no effect at 6 h, but a doubling in cell surface Pgp at 12 h after MMC treatment. Pgp levels as detected by both methods decreased subsequently to approximately half that of controls by 24 h after MMC and remained low until the end of the time course, 72 h after MMC treatment (Figure 4). Thus the basic response of GFP–Pgp to MMC in these cells was similar to that observed previously for native Pgp in H4IIE (Figure 1) and other cell lines, although the overall time course for this phenomenon was more rapid in the MDR29 cells. This seems to be a cell-type-specific phenomenon in which the kinetics of Pgp biogenesis is slightly different in each cell line. The temporal discrepancy between the Western and flow cytometry Pgp measurements suggests that there was a net movement of Pgp towards the plasma membrane by 6 h but that Pgp had not been fully inserted into the membrane in a functional orientation until 12 h after MMC treatment.

The functional consequences of these treatments were examined by measuring daunorubicin accumulation in MDR29 cells after treatment with MMC. As shown in Figure 5(A), there was a significant decrease in daunorubicin accumulation in cells treated for 12 h with MMC; drug levels in both control and MMC-treated cells were sensitive to verapamil, as predicted from the results in Figure 4 in which membrane Pgp levels had increased. Conversely, there was a verapamil-sensitive increase in drug accumulation at 48 h after MMC in treated compared with control cells (Figure 5B), which is consistent with a MMC-induced decrease in functional cell-surface Pgp expression at 48 h, as shown in Figure 4.

In contrast with the effects of MMC on Pgp expression, treatment of MDR29 cells with the anthracycline doxorubicin resulted in a progressive and substantial increase in membrane Pgp levels over a 24–72 h period after treatment (Figure 6). Cellular GFP levels were increased earlier and to a greater extent

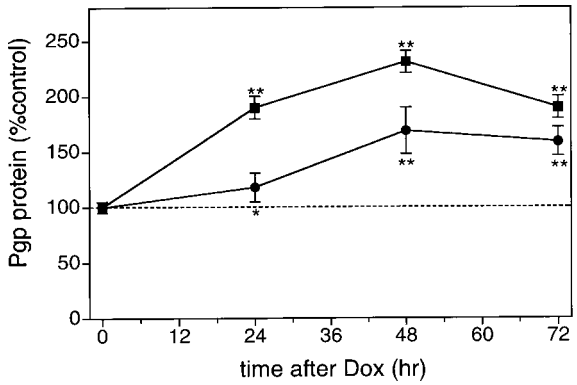

Figure 6 Effects of doxorubicin on total cellular and membrane-associated GFP–Pgp expression in MDR29 cells

Cells were treated with 1 μM doxorubicin (Dox) for 4 h and then analysed for GFP–Pgp levels by flow cytometry as described in the Experimental section and in the legends to Figures 3 and 5, with the use of the BD PGP antibody to detect membrane-associated human Pgp (●) or GFP fluorescence to detect total cellular GFP expression (■). Results are expressed as percentages of the corresponding control value at each time point and are means ± S.E.M. for values from three individual cultures (10000 cells were counted per sample). Asterisks denote values that were significantly different from the corresponding control: $^{*}P < 0.05$, $^{**}P < 0.01$; Student's t test.

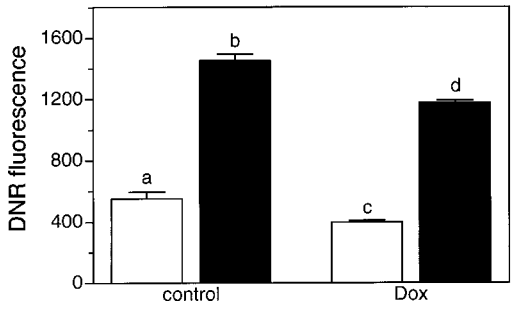

Figure 7 Effects of doxorubicin on drug accumulation in MDR29 cells

Cells were treated with doxorubicin for 48 h; daunorubicin (DNR) accumulation was measured in control cells (solvent, 48 h) or doxorubicin-treated cells (1 μM, 48 h after treatment) in the absence (open bars) or presence (filled bars) of verapamil by flow cytometry as described in the legend to Figure 4 and in the Experimental section. Results are means ± S.E.M. for determinations from three individual cultures per treatment (a minimum of 10000 cells were counted per sample). Bars labelled with different letters denote values that were significantly different from each other ($P < 0.05$, Student's t test).

than Pgp membrane levels after treatment with doxorubicin, indicating an initial increase in total cellular GFP–Pgp protein expression that was subsequently followed by an increase in plasma-membrane-associated GFP–Pgp. This is consistent with many previous reports demonstrating that treatment of Pgp-expressing cells with doxorubicin and other anthracyclines leads to an increase in MDR1 mRNA expression and Pgp protein expression [24]. As shown in Figure 7, doxorubicin caused a significant decrease in daunorubicin accumulation in MDR29 cells at 48 h, as predicted by the increase in cell surface Pgp shown in Figure 6. In summary, doxorubicin caused an increase in total cellular GFP–Pgp expression which led to an increase in functional membrane GFP–Pgp expression. In contrast, MMC caused an initial increase in functional membrane GFP–Pgp expression in the absence of increased total cellular GFP–Pgp expression, indicating a rapid and substantial increase in the net movement of GFP–Pgp to the cell surface in response to this drug.

DISCUSSION

Acquired drug resistance is a significant clinical issue in human cancers. In breast cancer, for example, newly diagnosed tumours that have not been pretreated with chemotherapy have little or no expression of Pgp and respond well to a variety of Pgp substrate drugs such as the anthracyclines and the taxanes [8,14–16]. In fact, the largest study so far conducted reported no detectable Pgp expression in 248 consecutive samples [16]. However, after repeated rounds of chemotherapy with a Pgp substrate agent such as doxorubicin, expression of Pgp was detected in 62% of all specimens examined [8,12,28]. The acquisition of a drug resistance phenotype is one of the primary reasons for the loss of responsiveness in late-stage tumours.

The *MDR1* gene coding for Pgp is an inducible gene whose expression can be modulated by drugs, hormones and other stimuli [17–19]. For example, the rat *mdr1* gene was demonstrated to be induced by treatment of rat hepatocytes with various DNA-damaging agents that form monoadducts, including simple alkylating agents such as methyl methanesulphonate, agents that form bulky monoadducts such as aflatoxin B_1 and 2-acetylaminofluorene, and with anthracyclines and their derivatives, such as doxorubicin and mitoxantrone [21,22,29]. It is important to note that, whereas cell lines selected for drug resistance in culture can overexpress Pgp by 50–100-fold or more, human cancers *in vivo* typically overexpress Pgp and other MDR-associated proteins by 2–5-fold, which is sufficient to confer a drug resistance phenotype [3,4]. Thus increases or decreases in the functional expression of Pgp of 2–5-fold in cell lines expressing Pgp at levels comparable with those of human MDR tumours would be expected to be directly clinically relevant.

We recently demonstrated that MMC treatment (0.1 μM for 4 h) of cancer cells that naturally overexpress Pgp at levels comparable with those in MDR cancers *in vivo* led to a significant decrease in MDR1 mRNA and subsequent Pgp protein expression. Pgp protein levels were at their lowest between 48 and 84 h after a single MMC treatment before recovering to control levels. This suppression occurred in both rodent and human cell lines, in those derived from tumours of the breast, colon, kidney, liver and brain and from acute leukaemia, and in cells grown either as adherent monolayers or as non-adherent spheroid cultures [12]. The primary target for the suppression of Pgp expression by MMC and other DNA cross-linking agents seemed to be the transcription of the *MDR1* gene; we have hypothesized that this is a direct or indirect result of the formation of DNA cross-links by these agents at or near the *MDR1* proximal promoter [12]. Similar effects of these agents on the transcription of another inducible gene, *PEPCK* (encoding phosphoenolpyruvate carboxykinase), were directly associated with specific regulatory elements within its promoter region [30,31].

In this study we have provided evidence for a second target for the effects of MMC on Pgp expression. This second step seems to involve the trafficking and/or maturation of nascent Pgp protein, because it does not involve a change in Pgp protein expression and because it results in a net movement of existing Pgp protein from an intracellular pool to the cell surface in response to treatment with MMC. Understanding the regulation of this step will be important in evaluating the overall MDR response of a tumour to chemotherapy. For MMC, a net movement of Pgp to the cell surface seems to be a futile response, because the dominant effect of this drug is to suppress Pgp mRNA expression,

leading to an overall decrease in Pgp protein expression at later times after drug treatment. It is therefore the combined effects on these two independent pathways that dictates the overall effects of a given drug on Pgp expression in tumour cells.

This phenomenon might also provide clues about the regulation of other gene products in this protein family, such as the related membrane ABC protein, CFTR, mutations of which are responsible for cystic fibrosis. In the case of CFTR, the ΔF508 mutation, which is present in 67% of all known CF patients, results in a decrease in expression of functional CFTR protein at the cell surface. This mutation is believed to result in a defect in CFTR folding and subsequent trafficking via the Golgi [32–34]. However, experimental cell culture procedures that aid in the folding and trafficking of this mutant to the cell surface also restore a normal phenotype [35–37], indicating that the folded and membrane-inserted ΔF508-CFTR protein functions normally. There may be one or more common steps in trafficking of these ABC proteins that is drug sensitive. Interestingly, other experiments in our laboratory have demonstrated that both MMC and doxorubicin increase the functional cell-surface expression of CFTR and that doxorubicin increases the functional cell-surface expression of ΔF508-CFTR in epithelial cells. This seems to be primarily through a post-transcriptional mechanism involving the net movement of CFTR to the membrane [38], suggesting a possible mechanism for reversing the ΔF508 phenotype by using systemic chemotherapy.

Our results suggest that MMC might be able to selectively increase one or more steps in post-transcriptional Pgp expression leading to functional membrane insertion. These might include changes in any step in the Pgp biosynthetic pathway. A recent study suggested that Pgp substrate drugs can act as 'chemical chaperones' to increase the cell-surface expression of folding mutants of Pgp comparable to that of the ΔF508-CFTR folding mutant [39]. It remains to be determined whether such a mechanism is responsible for the effects that we have observed. However, it should be noted that MMC is not a substrate (or is only a very poor substrate) for Pgp, yet it seems to be highly effective at increasing the net movement of Pgp to the cell surface. Moreover, doxorubicin, a Pgp substrate, increased the overall expression of Pgp but did not seem to affect its net movement to the membrane. It might be that both phenomena are occurring, i.e. Pgp substrate drugs might selectively aid in the folding of mutant or misfolded Pgp proteins, whereas a larger class of cytotoxic drugs might (also) be able to increase the trafficking of Pgp to the cell surface. We hypothesize that this latter phenomenon is a specific acquired regulatory step, which serves as a protective response of cells to cytotoxic xenobiotic challenge. The precise mechanistic basis for these effects remains to be determined.

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