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Functional studies with a full-length P-glycoprotein cDNA encoded by the Hamster pgp1 gene

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Abstract. Hamster cells grown in culture may, like human and mouse cells, develop multidrug resistance (MDR) when exposed to certain cytotoxic chemotherapeutic agents. Several phenotypic features that are characteristic of MDR have been described; these include (1) resistance to many structurally and functionally unrelated drugs that have different cellular targets and modes of action; (2) reversal of MDR by certain agents, including verapamil and cyclosporin A; and (3) reduced intracellular drug accumulation relative to that of drug-sensitive cells. In this report we show that the introduction and overexpression of the hamster pgp1 cDNA confers to otherwise drug-sensitive cells an MDR phenotype with these features. Moreover, pgp1 transfectants showed varying degrees of resistance to anthracycline analogues, indicating that structural analogues of commonly used anticancer agents are capable of circumventing drug resistance conferred by pgp.

Introduction

Human and rodent tumor cells have the capacity to acquire or intrinsically display resistance to the lethal effects of chemotherapeutic agents [13, 23]. One mechanism of resistance that has been studied extensively is multidrug resistance (MDR) conferred by P-glycoprotein (pgp) [11, 13, 23, 28]. Pgp is a 150- to 180-kDa plasma membrane transporter thought to act as an adenosine triphosphate (ATP)-dependent drug-efflux pump with broad substrate specificity. By maintaining intracellular drug concentrations below cytotoxic levels, pgp is thought to confer resistance to a variety of antineoplastic drugs, including actinomycin, daunorubicin, vincristine, and taxol, as well as many others [11, 13, 23, 28].

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Several lines of evidence support a model whereby pgp functions as an ATP-dependent drug-efflux pump that is capable of conferring mdr:

1. Pgp overexpression is tightly associated with MDR in tumor cell lines; resistant cells often overexpress pgp, whereas drug-sensitive parental and revertant cells do not [22, 24, 31, 32].
2. Pgp gene amplification and/or mRNA expression levels correlate well with drug resistance [17, 31, 34, 36, 37].
3. Pgp sequences inferred from pgp/MDR cDNAs indicate the presence of features characteristic of membrane transport proteins [4, 6, 8, 16, 18, 42].
4. Pgp contains two putative adenosine triphosphate (ATP)-binding sites that are essential for its activity [2].
5. The introduction and overexpression of MDR/pgp cDNAs confers MDR to otherwise drug-sensitive cells [6, 7, 15, 27, 40].
6. Such transfectant cells also maintain low intracellular drug concentrations relative to those of normal cells and otherwise resemble MDR cells [5, 19].

Although pgp is associated with the MDR in hamster cells [7, 8, 36] a demonstration that the hamster pgp1 gene product can confer an MDR phenotype with all of the properties mentioned above has thus far not been reported. Since many early studies on MDR were initially performed in hamster cells, a large body of unique information exists on the features of this phenomenon in this species [3, 20, 25, 26, 29]. In this report we show that the introduction and overexpression of the hamster pgp1 cDNA confers to otherwise drug-sensitive cells an MDR phenotype with features closely resembling those reported previously for drug-selected hamster MDR cell lines [3, 25]. Such transfectants were resistant to multiple agents, including actinomycin D, colchicine, vincristine, and anthracyclines, and resistance was reversed by two agents known to reverse pgp-mediated MDR, namely, verapamil and cyclosporin A. Moreover, transfectant cells expressing pgp1 showed reduced accumulation of a pgp substrate, colchicine, as compared with drug-sensitive control cells. Hence, like mouse mdr1 [15] and mdr3 [6] and human mdr1 [27, 40], the hamster pgp1 gene confers phenotypic features similar

to those observed in drug-selected cells expressing endogenous pgp [3, 20, 25, 26, 29].

We also demonstrate that pgp1 transfectants show varying degrees of resistance to a number of anthracycline analogues. Although pgp1 transfectants are highly resistant to some of these compounds, they show very low levels of resistance to others, indicating that certain anthracyclines might be extremely useful in the treatment of tumors resistant due to pgp expression.

Materials and methods

Cells. All cell lines used in this study were derived from the Chinese hamster lung cell line, DC-3F, developed by Biedler et al. [3, 26]. Cells were maintained in MEM/F12 (Gibco) media containing 5% fetal calf serum, which was heated to 56° C for 30 min prior to its use. Media also included 100 U penicillin/streptomycin per milliliter (Gibco).

Cellular accumulation of [^{3}H]-colchicine. Three cell lines were examined in parallel in a single experiment: DC-3F (parental), an antisense pgp transfectant, and a normal pgp transfectant (see below). Each cell line was grown to a density of approximately 1.0×10^6 cells in 60-mm tissue-culture plates. [^{3}H]-Colchicine was added to duplicate plates at a concentration of 30 ng/ml (10 ng labeled colchicine/ml + 20 ng unlabeled colchicine/ml) in MEM/F12 media containing 5% fetal calf serum and penicillin/streptomycin antibiotics. At various times of incubation with labeled drug at 37° C, the media were aspirated and the cells were quickly washed with ice-cold phosphate-buffered saline. The cells were then removed by trypsin and collected in ice-cold MEM/F12 media by centrifugation at 4° C. The pellet was solubilized in 2% sodium dodecyl sulfate (SDS) and the radioactivity associated with the cells was determined by scintillation counting.

Cloning, sequencing, PCR, synthesis of primers, and plasmid preparations. Cloning of appropriate restriction fragments was performed using standard ligation and cloning techniques [35]. Constructs were routinely confirmed by sequencing and/or restriction mapping. Sequencing was performed with a Sequenase kit (US Bichemical) using double-stranded templates denatured by treatment with 2 *N* sodium hydroxide. The polymerase chain reaction (PCR) was performed using a Cetus PCR kit and thermocycler. Primers used for sequencing and PCR either were homologous to T7 and SP6 promoter sequences adjacent to the multicloning site in pGEM4Z (Promega) or were custom designed and synthesized on an Applied Biosystems PCR-mate oligonucleotide synthesizer as described elsewhere [8]. Plasmids were prepared by the alkaline lysis method and banded in CsCl gradients as previously described [1].

Construction of transfectant cell lines. Stably transfected DC-3F cell lines were established by the $CaPO_4$ precipitate method [1]. Cells transfected with appropriate constructs (see below) were exposed to 60 ng methotrexate (MTX)/ml [4 times the concentration lethal to 50% of the parental DC-3F cells relative to untreated controls (ED_{50})] in MEM media containing antibiotics and 5% dialyzed fetal calf serum, which was heated to 56° for 30 min prior to its use. Colonies emerging after about 2 weeks in culture with MTX were expanded and cloned by limiting dilution in 96-well plates [12]. Clones were evaluated for resistance to vincristine, and those capable of displaying resistance to both MTX and vincristine were chosen for further study. Clonal stocks were not exposed to vincristine or any of the other drugs known to be common substrates for pgp. Several clones selected at 60 ng MTX/ml were exposed to higher concentrations of MTX, i.e., 120, 300, 600, 1,000, 1,500, 3,000, 6,000, 10,000, and 15,000 ng/ml, in an attempt to select indirectly cells expressing high levels of pgp from the vector. Colonies emerging from such reselections at a number of MTX concentrations were expanded, cloned, and evaluated for drug resistance; large increases in resistance to MDR drugs were not observed in such lines (data not shown). However, small increases (about 2- or 3-fold) relative to the resistance of clones selected at 60 ng/ml occurred in some clones selected at 300 ng/ml; further increases were not observed with increasing MTX concentrations (data not shown).

Drugs. Actinomycin D, colchicine, daunorubicin, and verapamil were obtained from Sigma. Vincristine sulfate was a gift from Eli Lilly. Doxorubicin, epirubicin, idarubicin, and 3′-deamino-3′-[2(S)-methoxy-4-morphylinyl] doxorubicin (DMM Dox) were gifts from Adria Laboratories and Farmitalia Erba. [^{3}H]-Colchicine was obtained from New England Nuclear. Cyclosporin A was obtained from Sandoz Pharmaceuticals. Methotrexate was a gift from Lederle Laboratories.

Drug assays. Drug resistance was evaluated in DC-3F cells and transfectants essentially as described elsewhere [26]. Cells were plated in 60-mm culture plates at a density of 4×10^4 cells/plate. Drugs were added 24 h after plating, and cytotoxicity was evaluated 72 h later. After the drug incubation period, cells were removed from the plates by trypsinization and then counted with a Coulter counter. Complete response curves were generated in triplicate for each drug examined; a range of drug concentrations resulting in 0–100% death of cells relative to untreated controls was tested. The ED_{50} was determined by linear regression analysis. The three independent ED_{50} measurements made for each drug in an experiment were averaged, and this value is reported with the standard error. Resistance was evaluated simultaneously for all drugs in each experiment shown.

Dot blots. Total cellular RNA was isolated by the guanidinium isothiocyanate method and pelleted by ultracentrifugation through a CsCl cushion as described elsewhere [1]. Dot blots and hybridization with the 4.3-kb full-length pgp1 cDNA probe [8] were performed as previously described [1].

Reversal assays. The drug-resistance assay described above was modified to include reversal agents. Toxicity due to addition of the individual reversal agent alone, i.e., either verapamil or cyclosporin A, was evaluated by generating complete toxicity curves for each agent in each cell line (data not shown). Concentrations just below those causing toxicity were chosen for reversal studies; in verapamil studies this was 1.0 μg/ml, whereas in cyclosporin A studies it was 2.5 μg/ml. For verapamil studies, phosphate-buffered saline solvent was used as a control, whereas in cyclosporin A studies, a solvent containing 1% ethanol and MEM/F12 was used to dissolve the drug and, hence, was also used as the solvent control; the solvents themselves were not toxic (data not shown). Reversal agents or solvent controls were added just prior to the addition of actinomycin D, colchicine, vincristine, or daunorubicin.

Western immunoblots. Western immunoblots were performed on total proteins obtained from cell membrane preparations using the C219 monoclonal antibody. Cell membranes were isolated and analyzed essentially as described elsewhere [9, 14]. In all cases 10 μg membrane protein was analyzed from each cell line. Samples were electrophoresed on a 7.5% SDS polyacrylamide gel without heating prior to loading as previously described [9, 14]. Duplicate gels were run; one gel was stained with Coomassie blue following electrophoresis to ensure that equivalent levels of pgp were loaded and analyzed. The second gel was transferred to nitrocellulose by electroblotting and was probed with the C219 antibody at a 1:500 dilution in PBST buffer (PBST contains 1× phosphate-buffered saline and 0.05% Tween-20). Signal was detected with a secondary anti-mouse antibody conjugated to horseradish peroxidase using hydrogen peroxide and luminol. Gels were exposed to Kodak XAR5 film for 1–10 min.

Results

Figure 1 shows a map of the full-length hamster pgp1 cDNA and the encoded pgp [8]. Like human and mouse pgps,

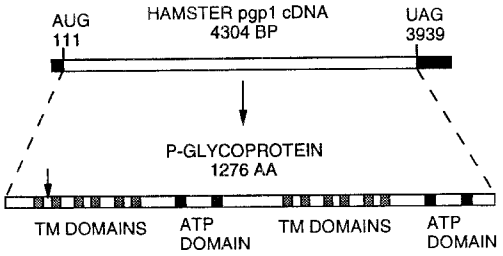

Fig. 1 Maps of hamster pgp1 cDNA and encoded pgp. The full-length hamster pgp1 cDNA is shown at the *top*, whereas the encoded pgp is shown at the *bottom*. *Top:* The full-length pgp1 cDNA represents a transcript of 4304 bases in length and contains both 5′ and 3′ untranslated regions (*black*) as well as a long open reading frame in the center (*white*) [8]. The AUG translational start codon at position 111 as well as the stop codon at 3939 are shown. *Bottom:* The 1276 amino acid pgp encoded by hamster pgp1 is shown [8]. Note the presence of 12 potential transmembrane domains (*hatched boxes*), 2 ATP-binding sites (*black boxes*), as well as potential glycosylation sites (*arrow*)

hamster pgp1 is predicted to contain 12 transmembrane domains, 2 ATP-binding sites, and glycosylation sites [8].

In prepartion for transfection studies, we first modified the full-length hamster cDNA by replacing the GC tails present at both 5′ and 3′ ends due to the initial cloning strategy with PCR adaptors containing *Bam*H I sites (Fig. 2). These manipulations were carried out in the plasmid pGEM4Z (Promega). Since the full-length pgp1 cDNA that we had originally cloned [8] had been found to contain mutations at codons 338 and 339 [7], we replaced this region with an equivalent fragment containing the wild-type sequence at these codons (kindly provided prior to publication by J. Endicott and V. Ling [10]). The resulting cDNA contained the full-length wild-type pgp1 sequence from base 1 to base 4304 and had *Bam*H I sites at either end. This insert was subsequently cloned into the *Bam*H I site of the expression plasmid pA3βT, which contains two eukaryotic promoters [21]; one drives the expression of a dominant-selectable dihydrofolate reductase (DHFR) marker, whereas the second promoter directs the expression of the pgp1 insert. The resulting pgp1 expression plasmids, containing the modified pgp1 cDNA insert in either the sense or the antisense orientation, were used to develop stably-transfected cell lines (see Materials and methods). Our inability to obtain transfectants with high levels of pgp expression by indirect selection with MTX and amplification of the vector, even though high levels of MTX resistance were obtained, most likely reflects presence of different mechanisms of MTX resistance that are not dependent upon DHFR overexpression and probably emerged during selection. Another possibility is that during the recombination steps that accompany amplification, the pgp1 insert was lost or inactivated.

Dot-blot experiments with RNA isolated from these tranfectants indicated that pgp RNA was overexpressed in the appropriate cell lines (Fig. 3A). In contrast, pgp RNA levels were not elevated in clones transfected with the vector alone as compared with the drug-sensitive parental DC-3F cell line. Northern blots also showed the overexpression of pgp transcripts of the expected size (4.3 kb in cells transfected with sense or antisense vectors but not in those transfected with the vector alone (data not shown).

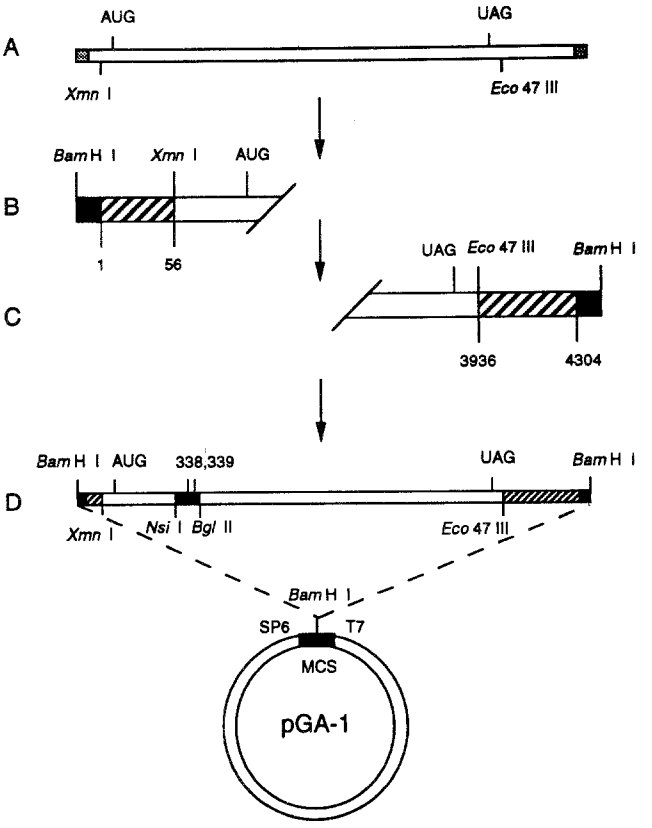

Fig. 2A–D. Modification of pgp1 cDNA for expression studies. **A** The full-length hamster pgp1 cDNA initially contained GC tails at either end as a result of the cloning strategy (*stippled boxes*) [8]. Since these might have been detrimental to expression and because no convenient restriction site was available at the ends of the cDNA, both ends were modified. The GC tails were removed and replaced with PCR adaptors containing *Bam*H I restriction sites positioned adjacent to the terminal bases of the cDNA. **B1** The 5′ end of the cDNA from the GC tail to the *Xmn* I restriction site at position 56 was removed and replaced with a PCR adaptor, which contained a *Bam*H I restriction site (*black*) adjacent to the first 56 bases of cDNA (*hatched*). This adaptor was constructed using the PCR; the strategy employed a primer containing a *Bam*H I restriction site and the first 20 bases of the pgp1 leader sequence, paired with a primer homologous to bases 36–56, and used the unmodified pgp1 cDNA as a template [8]. **C** A similar strategy was used to modify the 3′ end, instead taking advantage of the unique *Eco*47 III restriction site at position 3936. **D** The modified cDNA was cloned into the *Bam*H I site of pGEM4Z (Promega) to generate the plasmid p4.3**, after which a unique *Nsi* I/*Bgl* II restriction fragment (*black*) was removed and replaced with an equivalent fragment containing the wild-type pgp1 sequence at codons 338 and 339 [10]. This plasmid, containing the pgp1 cDNA from bases 1 to 4304 in the *Bam*H I site, was named pGA-1. The modified *Bam*H I pgp1 insert of pGA-1 was subsequently cloned into the *Bam*H I site of the eukaryotic expression plasmid pA3βT [21] downstream of the human β-actin promoter, which drives the expression of this insert.

Pgp was likewise overexpressed appropriately in transfectants as determined by Western immunoblots with either total cell lysates (data not shown) or membrane preparations using the C219 monoclonal antibody [24] (Fig. 3B). Finally, Southern blots showed the acquisition of the 4.3-kb pgp insert as well as pBR322 vector sequences, as expected (data not shown).

Complete drug-response curves generated for these transfectants indicated that each of the clones over-

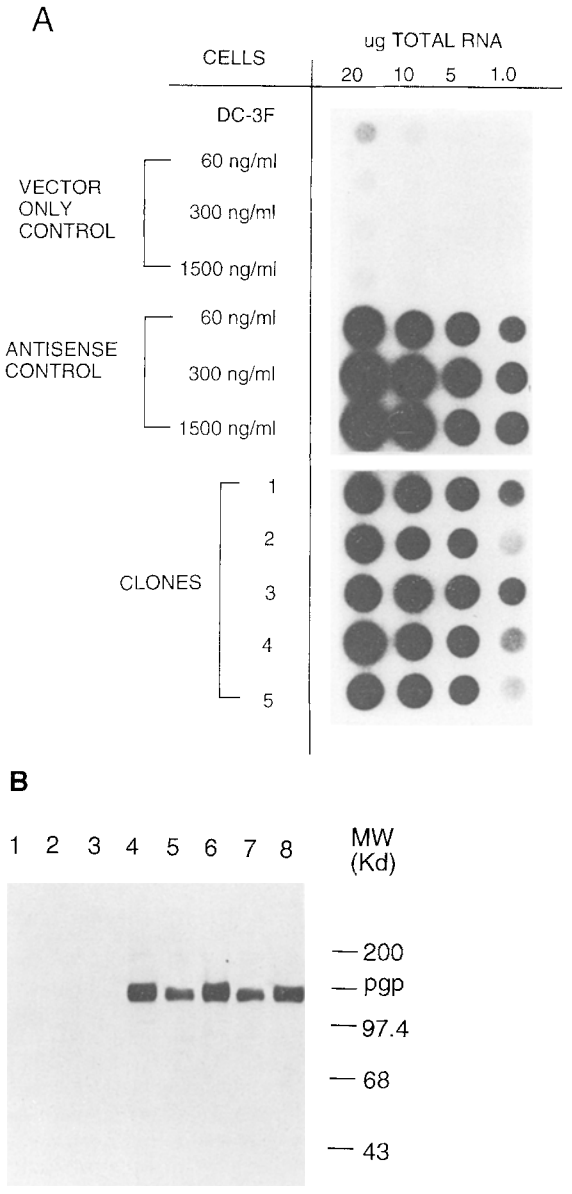

Fig. 3A–B. Analysis of pgp RNA and protein in transfectant clones. **A** RNA dot-blot analysis of transfectant clones. The vector alone or constructs containing the pgp1 cDNA in either the antisense or sense orientation were introduced into drug-sensitive hamster DC-3F cells, and clones containing a construct were selected using MTX (See Materials and methods). Total RNA (amount indicated) was applied to nitrocellulose and hybridized with the 4.3-kb *Bam*H I insert of pGA-1 (Fig. 2) as described elsewhere [35]. Clones containing the vector alone, which were selected at a range of MTX concentrations, showed no increase in pgp RNA. On the other hand, antisense controls, which were also selected at different MTX concentrations, showed elevated pgp RNA, in agreement with the expression of antisense transcripts that would hybridize to the pgp1 ds cDNA probe. Finally, each of five clones containing a sense pgp1 construct expressed elevated levels of pgp mRNA. **B** Western immunoblot obtained using the C219 monoclonal antibody [24]. In all, 10 μg crude membrane proteins was analyzed from each clone. *Lane 1*, DC-3F; *lane 2*, vector control; *lane 3*, antisense control; *lanes 4–8*, clones transfected with the sense construct. Note the pgp overexpression in lanes 4–8 at 150–180 kDa. Each clone shown was selected and maintained at 300 ng/ml MTX, thus maintaining selective pressure for the presence of the vector. The transfer of prestained molecular-weight markers to nitrocellulose was monitored to ensure complete transfer; also, Coomassie blue staining of such gels following electroblotting routinely confirmed uniform and complete transfer

Table 1. Drug resistance in transfectant clones

	Fold resistance relative to DC-3F[a]			
Clone	Act D	Colch	VCR	DR
Vector only	1.3±0.1	0.97±0.02	1.4±0.4	1.8±0.1
Anti-sense	1.7±0.02	1.5±0.01	1.5±0.1	1.0±0.2
1[b]	50.4±3.6	113.5±7.5	92.3±5.2	72.7±1.7
2	33.1±5.0	58.4±0.9	66.3±6.6	43.0±11.6
3	28.0±11.2	68.0±6.8	66.1±4.1	62.9±12.1
4	43.9±0.7	101.2±10.0	113.0±16.0	40.5±1.6
5	44.1±0.8	77.3±3.1	73.4±4.7	40.1±2.6

Act D, Actinomycin D; Colch, colchicine; VCR, vincristine; DR, daunorubicin

[a] Each drug batch was first calibrated in parental DC-3F cells to the following values: actinomycin D, 3.0 ng/ml; colchicine, 30 ng/ml; vincristine, 100 ng/ml; and daunorubicin, 15 ng/ml. Baseline values for DC-3F can vary by approximately ± 0.2- to 0.8-fold in independent experiments

[b] The values shown for clone 1 were obtained from the full drug-response curves shown in Fig. 4. All values listed were obtained from curves generated in this manner

Table 2. Reversal of MDR by verapamil or cyclosporin A

		Fold resistance relative to DC-3F[a]			
Experiment[b]		Act D	Colch	VCR	DR
Verapamil:					
1.	–	36.1±14.3	70.2±2.1	69.4±4.4	36.4±0.9
	+	1.2±1.0	11.2±0.9	6.5±0.9	2.8±0.2
2.	–	48.6±4.4	80.9±5.2	98.0±1.2	42.5±1.4
	+	2.1±0.5	15.1±0.5	5.3±0.3	2.7±0.1
Cyclosporin A:					
3.	–	33.8±10.7	66.3±2.9	45.4±1.2	65.0±1.6
	+	5.1±0.9	2.1±0.4	3.6±0.2	3.1±0.1
4.	–	47.1±3.6	71.0±4.3	63.7±6.3	56.5±2.8
	+	4.0±0.5	2.4±0.1	2.1±1.0	2.2±0.9

[a] The batches of drugs used in these experiments were the same as those listed in Table 1

[b] Clone 2 was used in each of these experiments

expressing pgp was resistant to multiple agents, including actinomycin D, colchicine, daunorubicin, and vincristine (Fig. 4, Table 1). Control transfectants, on the other hand, showed no drug resistance (parental DC-3F, vector-only, and antisense controls all gave similar results; Table 1). Full drug-response curves are shown for one pgp1 transfectant in Fig. 4, and resistance values obtained from similar curves are listed for the five different pgp transfectants in Table 1. These clones showed resistance levels ranging from 25- to 120-fold resistance to these agents.

We next determined whether the MDR displayed by the transfectants could be reversed by verapamil and/or cyclosporin A, two agents known to reverse MDR in drug-selected cell lines expressing endogenous pgps [39]. Importantly, both reversal agents reduced the level of resistance conferred to each of the four drugs tested, namely, actinomycin D, colchicine, vincristine, and daunorubicin (Table 2). Hence, in a manner similar to that observed in

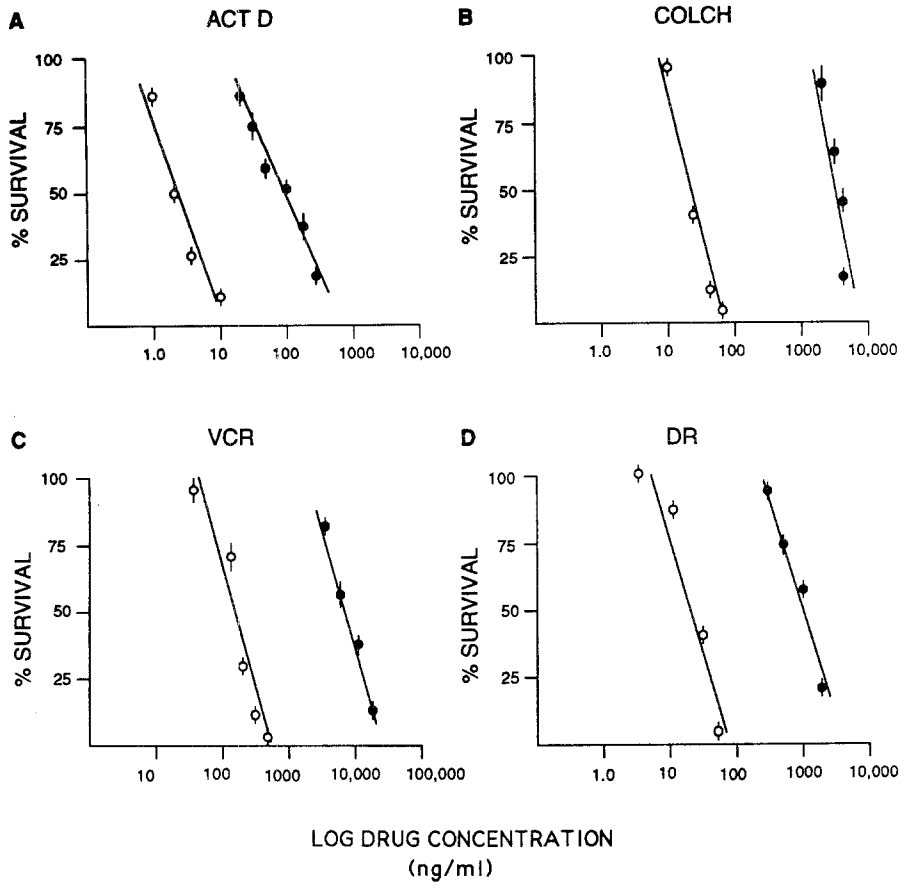

Fig. 4A–D. Drug-response curves constructed for a pgp1 transfectant expressing pgp. Drug-response curves were generated for each of four different drugs. Cells were tested over a range of drug concentrations that led from 0 to 100% cell death. Each drug concentration was tested in triplicate, and the data points plotted represent the mean of the triplicate measurements; error bars indicate the standard deviation. **A** Drug-response curves generated for actinomycin D: antisense control clone (*open circles*) and a pgp1 transfectant overexpressing pgp (*filled circles*). **B** Drug-response curves constructed for colchicine. **C** Drug-response curves obtained for vincristine. **D** Drug-response curves generated for daunorubicin. In each case the percentage of survival relative to cells receiving no drug was plotted on the Y-axis, whereas the log of the drug concentration (in nanograms/ml) was plotted on the X-axis

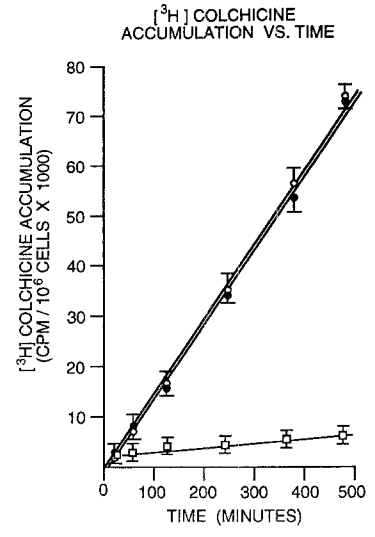

Fig. 5. [^{3}H]-Colchicine accumulation in pgp transfectants. Accumulation of [^{3}H]-colchicine was examined in the parental DC-3F cell line (*open circles*), the antisense control (*filled circles*), and a pgp1 transfectant overexpressing pgp (*open squares*). A representative experiment is shown in which the average values of triplicate determinations have been plotted at each point. Similar results were obtained in four independent experiments

hamster, human, and mouse cells selected directly with drugs and displaying MDR, these agents were also capable of effectively reversing the MDR conferred by the transfection of pgp1. Moreover, all four drugs were reversed, indicating that the sites on hamster pgp1 responsible for drug reversal by verapamil and cyclosporin A bear relationships to the site or sites involved with conferring resistance to each of the drugs studied.

An early observation concerning the mechanism of MDR was that hamster cells selected with actinomycin D, colchicine, or daunorubicin showed decreased intracellular accumulation of these compounds relative to that of drug-sensitive parental cells [3, 25, 30]. Thus, we tested whether accumulation of one of these agents, colchicine, was altered in pgp1 transfectants. Indeed, transfectants expressing the pgp1 cDNA showed approximately 10-fold lower levels of colchicine accumulation relative to that observed in controls (Fig. 5). Hence, hamster pgp1 confers upon cells the ability to maintain low intracellular colchicine concentrations, in complete agreement with observations made of drug-selected hamster cells overexpressing endogenous pgp [25].

Finally, we tested the resistance of these pgp1 transfectants to a number of anthracycline analogues. Interestingly, the extent of drug resistance observed was dependent upon the analog used, and the same relative patterns were observed in two different clones. Resistance was highest to epirubicin, followed by moderate levels of resistance to daunorubicin and doxorubicin, low levels of resistance to idarubicin, and very low levels of resistance to DMM Dox (Table 3). These data indicate that the different analogues differ greatly as potential substrates for pgp1. Moreover, both DMM Dox and idarubicin, which are poor substrates for hamster pgp1, may be very useful in the treatment of tumors resistant to elevated pgp expression. This is especially true for DMM Dox, since this compound retains

Table 3. Resistance of anthracycline analogues

Analogue	Fold resistance relative to DC-3F[a]	
	Clone 3	Clone 5
Epirubicin	105.7±11.5	48.0±1.4
Daunorubicin	62.9±12.1	40.1±2.6
Doxorubicin	57.4±8.1	35.5±3.8
Idarubicin	17.5±2.7	14.2±1.0
DMM Dox	2.5±0.5	3.5±1.6

[a] The ED_{50} values for these compounds in parental DC-3F cells were as follows: epirubicin, 45.5±0.7 ng/ml; daunorubicin, 18.8±1.2 ng/ml; doxorubicin, 42.5±3.3 ng/ml; idarubicin, 2.5±0.2 ng/ml; DMM Dox, 23.2±2.7 ng/ml

potent antitumor activity [33] but is capable of almost completely evading the pgp mechanism of resistance observed for other anthracyclines.

Discussion

Some of the earliest studies of the phenomenon of MDR employed drug-resistant hamster cells, and such cells were found to possess a number of characteristics now known to be common to the MDR phenotype [3, 20, 25, 26, 29, 38]. These included cross-resistance to unrelated compounds, the maintenance of low intracellular drug concentrations, and phenotypic reversal by verapamil and other agents. Our studies indicate that the overexpression of the glycoprotein encoded by the hamster pgp1 gene is sufficient to confer a phenotype with these features.

Early studies with drug-selected hamster cell lines indicated that resistance was due to decreased drug accumulation in resistant cells [25, 30, 35]. For example, the nuclei of actinomycin D-resistant cells were not as highly labeled with tritiated actinomycin D as were the nuclei of normal cells; these results suggested that an alteration in membrane permeability might have led to this drug resistance [3]. Colchicine accumulation was shown to be similarly altered in resistant hamster cells selected with colchicine [25]. This decreased accumulation was energy-dependent, leading the authors to conclude that a drug barrier that was dependent upon cellular energy for function was present in the membranes of resistant cells [25]. The results presented in this report indicate that the introduction and overexpression of the hamster pgp1 cDNA is sufficient to confer this phenotype of reduced colchicine accumulation.

Four groups of investigators independently cloned segments of hamster pgp genes or cDNAs and used these probes to clone human, mouse, and hamster genes and cDNAs [4, 6, 8, 16–18, 31, 34, 36, 41, 42]. Nevertheless, direct proof that hamster pgp genes were responsible for conferring the MDR phenotypes in these cells was lacking. The results of transfection experiments shown herein using the full-length pgp1 cDNA, however, directly confirm that this gene is capable of conferring an MDR phenotype with features similar to those found in MDR hamster cells.

cDNAs representing two human mdr/pgp genes and three mouse genes have been reported, and each of these has been tested in transfection experiments for its ability to confer MDR. In each species, one or more members of the multigene family were found to be capable of conferring MDR, whereas a single gene-family member in each species was inactive. For example, in the mouse, the mdr1 and mdr3 genes confer MDR, whereas the mdr2 gene does not. The hamster pgp1 inferred amino acid sequence is most closely related to the biologically active mouse mdr3, showing 93% identity to this sequence [8]. Hence, on the basis of homology alone, pgp1 would be predicted to be a functional member of the hamster pgp gene family. Transfection experiments presented in this report confirm that pgp1, in fact, encodes a functional pgp with features similar to those displayed by functional members of the human and mouse mdr gene families. Indeed, given the strong amino acid sequence homology between mouse mdr3 and hamster pgp1, one might expect that they would mediate very similar, if not the same, cross-resistance patterns. An assessment of that possibility requires that both be transfected into the same recipient cells such that cell-type variation in the extent of posttranslational modification of pgp can be ruled out. However, since it is known that single amino acid differences in pgps can dramatically alter cross-resistance patterns [13], it is not clear that mdr3 and pgp1 would yield the same result.

Finally, we showed that certain anthracycline analogues are very good pgp substrates, whereas others are very poor pgp substrates. Such data indicate that structural analogues of commonly used anticancer agents are capable of circumventing drug resistance conferred by pgp. Moreover, transfectant cell lines engineered to overexpress pgp specifically are useful tools for the identification of such compounds.

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References

1. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JS, Struhl K (eds) (1989) Current protocols in molecular biology. Greene, New York, pp 1.7.1, 4.0.1, 9.1.1
2. Azzaria M, Schurr E, Gros P (1989) Discrete mutations introduced in the predicted nucleotide-binding sites of the mdr1 gene abolish its ability to confer multidrug resistance. Mol Cell Biol 9: 5289
3. Biedler JL, Riehm H (1970) Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic, and cytogenetic studies. Cancer Res 30: 1174
4. Chen C-J, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM, Roninson IB (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell 47: 381
5. Croop J, Guild B, Gros P, Housman D (1987) Genetics of multidrug resistance: relationship of cloned gene to the complete multidrug resistant phenotype. Cancer Res 47: 5982
6. Devault A, Gros P (1990) Two members of the mouse mdr gene family confer multidrug resistance with overlapping but distinct drug specificities. Mol Cell Biol 10: 1652

7. Devine SE, Ling V, Melera PW (1992) Amino acid substitutions in the sixth transmembrane domain of P-glycoprotein alter multidrug resistance. Proc Natl Acad Sci USA 89: 4564
8. Devine SE, Hussain A, Davide JP, Melera PW (1988) Full-length and alternatively spliced pgp1 transcripts in Chinese hamster lung cells. J Biol Chem 266: 4545
9. Dhir R, Gros P (1992) Functional analysis of chimeric proteins constructed by exchanging homologous domains of two P-glycoproteins conferring distinct drug resistance profiles. Biochemistry 31: 6103
10. Endicott JA, Sarangi F, Ling V (1991) Complete cDNA sequences encoding the Chinese hamster P-glycoprotein gene family. DNA Sequences 2: 89
11. Endicott JA, Ling V (1989) The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Rev Biochem 58: 137
12. Freshney RI (1983) Culture of animal cells, a manual of basic technique. Alan Liss, New York, p 129
13. Gottesman MM, Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62: 385
14. Greenberger LM, Williams SS, Horwitz SB (1987) Biosyntheses of heterogeneous forms of multidrug resistance-associated glycoproteins. J Biol Chem 262: 13685
15. Gros P, Ben Neriah Y, Croop J, Housman DE (1986) Isolation and expression of a complementary DNA that confers multidrug resistance. Nature 323: 728
16. Gros P, Raymond M, Bell J, Housman D (1988) Cloning and characterization of a second member of the mouse mdr gene family. Mol Cell Biol 8: 2770
17. Gros P, Croop J, Roninson I, Varshavsky A, Housman DE (1986) Isolation and characterization of DNA sequences amplified in multidrug-resistant hamster cells. Proc Natl Acad Sci USA 83: 337
18. Gros P, Croop J, Housman D (1986) Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell 47: 371
19. Hammond JR, Johnstone RM, Gros P (1989) Enhanced efflux of [^{3}H]vinblastine from Chinese hamster ovary cells transfected with a full-length complementary DNA clone for the mdr1 gene. Cancer Res 49: 3867
20. Howell N, Belli TA, Zaczkiewicz H, Belli JA (1984) High level, unstable Adriamycin resistance in a Chinese hamster mutant cell line with double minute chromosomes. Cancer Res 44: 4023
21. Hussain A, Lewis D, Yu M, Melera PW (1992) Construction of a dominant selectable marker using a novel dihydrofolate reductase. Gene 112: 179
22. Juliano RL, Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta 455: 152
23. Kartner N, Ling V (1989) Multidrug resistance in cancer. Sci Am 260: 44
24. Kartner N, Evernden-Porelle D, Bradley G, Ling V (1985) Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. Nature 316: 820
25. Ling V, Thompson LH (1974) Reduced permeability in CHO cells ad a mechanism of resistance to colchicine. J Cell Physiol 83: 103
26. Melera PW, Biedler JL (1991) Molecular and cytogenetic analysis of multidrug resistance-associated gene amplification in Chinese hamster, mouse sarcoma, and human neuroblastoma cell. In: IB Roninson (ed) Molecular and cellular biology of multidrug resistance in tumor cells. Plenum, New York, p 117
27. Pastan I, Gottesman MM, Ueda K, Lovelace E, Rutherford AV, Willingham MC (1987) A retrovirus carrying an mdr1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. Proc Natl Acad Sci USA 85: 4486
28. Pastan I, Gottesman MM (1991) Multidrug resistance. Annu Rev Med 42: 277
29. Peterson RHF, Meyers MB, Spengler BA, Biedler JL (1983) Alteration of plasma membrane glycopeptides and gangliosides of Chinese hamster cells accompanying resistance to daunorubicin and vincristine. Cancer Res 43: 222
30. Riehm H, Biedler JL (1971) Cellular resistance to daunomycin in Chinese hamster cells in vitro. Cancer Res 31: 409
31. Riordan JR, Deuchars K, Kartner N, Alon N, Trent J, Ling V (1985) Amplification of P-glycoprotein in multidrug-resistant mammalian cell lines. Nature 316: 817
32. Riordan JR, Ling V (1979) Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. J Biol Chem 254: 12701
33. Ripamonti M, Pezzoni G, Pesenti E, Pastori A, Farao M, Bargiotti A, Suaroto A, Spreafico F, Grandi M (1992) In vivo anti-tumour activity of FCE 23762, a methoxymorpholinyl derivative of doxorubicin active on doxorubicin-resistant tumour cells. Br J Cancer 65: 703
34. Roninson IB, Abelson HT, Housman DE, Howell N, Varshavsky A (1984) Amplification of specific DNA sequences correlates with multi-drug resistance in Chinese hamster cells. Nature 309: 626
35. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Press, Cold Spring Harbor, New York, p 1.53
36. Scotto KW, Biedler JL, Melera PW (1986) Amplification and expression of genes associated with multidrug resistance in mammalian cells. Science 232: 751
37. Shen D-W, Fojo A, Chin JE, Roninson IB, Pastan I, Gottesman MM (1986) Human multidrug-resistant cell lines: increased mdr1 expression can precede gene amplification. Science 232: 643
38. Subak-Sharpe H (1965) Biochemically marked variants of the Syrian hamster fibroblast cell line BHK21 and its derivatives. Exp Cell Res 38: 106
39. Tsuruo T (1991) Reversal of multidrug resistance by calcium channel blockers and other agents. In: Roninson IB (ed) Molecular and cellular biology of multidrug resistance in tumor cells. Plenum, New York, p 349
40. Ueda K, Cardarelli C, Gottesman MM, Pastan I (1987) Expression of a full-length cDNA for the human mdr1 gene confers resistance to colchicine, doxorubicin, and vinblastine. Proc Natl Acad Sci USA 84: 3004
41. Van der Bliek AM, Van der Velde-Koerts T, Ling V, Borst P (1986) Overexpression and amplification of five genes in a multidrug-resistant Chinese hamster ovary cell line. Mol Cell Biol 6: 1671
42. Van der Bliek AM, Kooiman PM, Schneider C, Borst P (1988) Sequence of mdr3 cDNA encoding a human P-glycoprotein. Gene 71: 401