Identification of P-glycoprotein Mutations Causing a Loss of Steroid Recognition and Transport

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P-glycoproteins transport a wide variety of hydrophobic compounds out of cells. While the diversity of transported molecules suggests a mechanism involving broad specificity, there is evidence of significant discrimination within given classes of molecules. One example of this behavior is transport of corticosteroids by the murine mdr1 P-glycoprotein. The presence of hydroxyl groups, associated with specific steroid carbon atoms, regulates the ability of corticosteroids to be transported. This specificity is demonstrated here by experiments measuring the ability of steroids to inhibit drug transport. The results indicate that a keto oxygen associated with the 3- and 20-carbon atoms, as well as a 17-carbon hydroxyl group, each acts to enhance steroidal P-glycoprotein inhibitory activity. Moreover, inhibitory steroids can be used for directed selection of variant cells, expressing mutated P-glycoproteins with a severely impaired ability to transport dexamethasone. The five mutations, reported here, are located within transmembrane domains 4–6, proximal to the cytoplasmic interface. The altered P-glycoproteins exhibit reduced capacity to be inhibited by specific steroids, suggesting decreased capacity to bind these molecules avidly. Studies comparing the relative inhibitory activity of a series of steroids indicate that these mutations alter recognition of the 17α-hydroxyl group and the 20-keto oxygen atom.

P-glycoprotein (Pgp)* expression causes increased resistance to dexamethasone-induced apoptosis in murine thymoma cells as well as resistance to a variety of other drugs (1). The likelihood that steroids are substrates for Pgp-mediated transport is supported by the observations that mdr1 P-glycoprotein expression leads to reduced intracellular dexamethasone accumulation and inhibition of hormone-dependent gene induction (2). Moreover, these effects are reversed by drug transport inhibitors, such as verapamil, RU486 (3, 4), and PSC-833. However, the phenomenon of Pgp-dependent steroid resistance is restricted at two levels. Glucocorticoid resistance is both gene-specific and steroid-specific. The dual specificity is exemplified by the following. 1) The mdr1, but not the mdr3, Pgp conveys resistance to dexamethasone (54). 2) The degree of resistance to a given steroid is highly dependent upon its structure. The degree of Pgp-dependent resistance to steroids that differ by only a single oxygen atom can be profoundly different. For instance, the presence of a 17α-hydroxyl group is associated with a greater shift in steroid sensitivity. Thus, the relative increase in resistance to cortisol is greater than for corticosterone, and the shift for dexamethasone greater than 17-desoxydexamethasone (1). An 11β-hydroxy group has an even greater effect. Corticosterone is nearly identical to cortisol but lacks an 11β-hydroxyl group. No evidence of increased resistance to corticosterone was observed, suggesting that it is not efficiently transported. Similarly, there is evidence that progesterone, which does not have any hydroxyl groups in its structure, is not transported (1, 5).

Progesterone has been shown capable of reversing Pgp-dependent drug resistance, inhibiting photoaffinity labeling of Pgp by azidopine and serving as a photoaffinity label of the human Pgp (5, 6). Therefore, it is evident that progesterone can bind to Pgp, even if it is not transported. In general, the relative ability of steroids to reverse drug resistance and inhibit drug binding to Pgp has been found to be highly dependent upon the hydrophobicity of the individual steroids (7, 8). This behavior suggests a lack of specificity in the interactions between the hormone and the protein but may also reflect the relative ability of the steroids to accumulate within the membranes containing the Pgp. The greater accumulation of the more hydrophobic molecules would be reflected in the local concentration of steroid available to interact with the Pgp. There is very little evidence indicating the region(s) of the Pgp that interact with steroids. One mutation, S941F, in the second half of the murine mdr1 Pgp (transmembrane domain 11), was found to affect the ability of progesterone to serve as an inhibitor of drug transport (9, 10). This mutation also caused a loss of adriamycin and colchicine resistance. There has been considerable effort invested in the characterization of drug resistance changes caused by Pgp mutations (11). Mutations that have arisen in cultured cells selected for resistance to specific drugs (12–14) and mutations introduced by site-directed mutagenesis have been evaluated (10, 15–32). In many cases, the resistance to one drug may increase while the resistance to others either changes or remains the same. Altering a given amino acid in multiple ways can produce profoundly different changes in the drug resistance profiles. In addition, trying to relate changes in resistance to altered drug binding can produce unanticipated results. The mutation G185V in the human Pgp causes increased colchicine resistance and decreased vinblastine resistance. However, this mutation was also reported to cause a decrease in colchicine binding affinity and an increase in vinblastine binding affinity (33). It would be very valuable to have a methodical means to identify mutations that alter the interaction of specific drugs with Pgp. The work presented here represents our progress in developing such an approach with steroid inhibitors. It focuses upon the isolation

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1 The abbreviations used are: Pgp, P-glycoprotein; 5αPdeo, 5α-pregnan-17α-ol-3,20-dione; RT, reverse transcriptase; PCR, polymerase chain reaction; bp, base pair(s).

2 D. Gruol, unpublished observation.
of cell lines expressing murine mdr1 Pgp that contain mutations that decrease the ability of steroids to act as transport inhibitors. The overall assumption is that the ability to inhibit drug transport primarily reflects the ability of the steroid to bind to the Pgp at a mechanistically important region of the protein. The results indicate that steroids interact with the Pgp at a site involving transmembrane domains 4–6. Moreover, the mutations appear to affect the protein’s interaction with specific steroid structural features.

**Experimental Procedures**

**Cell Culture—**WEHI-7 is a thymoma cell line obtained from a female BALB/c mouse after exposure to x-irradiation (34). W7TB is a derivative of WEHI-7 and is resistant to bromodeoxyuridine. Bromodeoxyuridine resistance is unrelated to multidrug resistance. MS23 is a variant selected from W7TB through prolonged growth in low levels of dexamethasone (35). HEK 293T is a human embryonal kidney epithelial line, transformed with the adenovirus E1a and SV40 large T antigen oncogenes. All of the cell lines were grown in suspension in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The incubator was maintained at 37 °C and had a humidified atmosphere of 13% CO₂ and 87% air.

**Selection of MS23 Variants—**Independent selections were initiated by the mutagenesis of MS23 cells with N-methyl-N-nitro-N-nitroso-guanidine, obtained from Sigma. This compound is an alkylating agent that predominantly causes point mutations. N-Methyl-N-nitro-N-nitroso-guanidine (2 μg/ml for 2 h) was used based on previous results with this compound and WEHI-7 cells (36). After allowing sufficient time for the cells to recover and express the mutated genes (usually 4–5 days), the cells were dispersed into multiwell dishes (1 × 10⁶ cells/well) in medium containing a toxic drug and the P-glycoprotein inhibitor 5β-pregnan-17α-ol-3,20-dione (5μPodo). Three independent mutagenesis/expression experiments have been carried out. In the first, the variant was selected in 3 μM puromycin and 20 μM 5μPodo. In the second and third selections, the variants were isolated from the combination of 3 μM puromycin and 10 μM 5μPodo. Resident colonies appeared between 12 and 18 days. Each of the variant lines was initially tested for changes in growth in the presence of dexamethasone (10 and 30 nM) relative to MS23 cells. The results of these evaluations identified those variants that had lost significant steroid resistance.

**Quantification of Drug Effects on Cellular Proliferation—**The effect of drugs on cell proliferation was measured as described previously (37). Briefly, cell cultures were set up in varied concentrations of drugs and incubated for 7 days. The amount of accumulated cellular material was assayed by measuring the turbidity of the cultures (660 nm) and by expressing the values as normalized to those from cultures grown in the absence of drug. These relative turbidity values reflect the amount of cellular material synthesized during the period of incubation and provide a sensitive measure of the capacity of the cells to proliferate, even if a large portion of them are killed. Typically, relative turbidity values of <5% represent situations where all of the cells have lost viability. The LC₅₀ value is defined as the concentration of drug that produces a relative turbidity value of 50%.

The relative ability of nonsteroid pregnanes to reverse P-glycoprotein-dependent drug resistance in cells was evaluated as follows. A series of cultures (5 × 10⁶ cells/ml) were grown with a fixed concentration of a toxic drug, to which cell line is normally resistant based upon its P-glycoprotein expression. Increasing concentrations of the pregnane were included in the culture medium, and the relative turbidity values of the cultures were evaluated after 7 days. The inhibitory efficiency of the pregnane is expressed by an EC₅₀ value defined as the concentration of chemosensitizer that reduces the relative turbidity value to 50%. The steroids used were obtained from Steraloids Inc. (Newport, RI). All steroids were assessed, by Steraloids, as a uniform single spot on TLC reaction (Life Technologies, Inc.). The sample was extracted with phenol/chloroform, and the cDNA was precipitated with ethanol. PCR amplification of the cDNA was carried out using a primer (P2) that overlaps another portion of exon 2, GGGAGAGGCGAGAACACTTCTCGAAGATG, and the primer (P3), GCTCTAGACTCGAGTCGAGCATCGTG. The PCR product was purified by agarose gel electrophoresis and purified by direct DNA sequencing from pMTmdr (pMDSN Sequencing System; Promega, Madison, WI). The sequencing reactions were subjected to electrophoresis on 6% polyacrylamide urea gels, and the resulting gels were analyzed by autoradiography.

**Evaluation of mdr1 P-glycoprotein Mutations Expressed in the MS23 Variants—**RT-PCR was used to generate a series of overlapping cDNA fragments encompassing the entire coding sequence of the mdr1 gene expressed in MS23 and the variant cell lines: MSPP-1, MSPP-7, MSPP-15, and MSPP-17. Five large overlapping cDNA fragments (A–E) were initially produced, and a series of subfragments (A₁–A₇, B₁–B₇, C₁–C₇, D₁–D₇) were generated using a set of nested primers. The primers used are shown in Table I.

The individual PCR products were purified by agarose gel electrophoresis and evaluated by direct DNA sequencing using the appropriate primers (5′/3′ DNA Sequencing System, Promega, Madison, WI) that had been end-labeled with ³²P. All primers were obtained from Life Technologies (Life Technologies, Inc.).

**Expression of Human Cells Expressing the Mouse mdr1 P-glycoproteins—**The murine mdr1 gene was introduced into a modified version of the pMT2 (40, 41) expression vector as described previously (54). The mdr1 gene in the pMTmdr vector was also modified to include the change G to T at nucleotide 692 of the coding sequence. A 1606-bp fragment of the mdr1 gene was removed from the pMTmdr vector using A/JII and ApoI and cloned into the Litmus 39 vector (New England Biolabs). The resulting construct (Lit39-mdr/AFaP) was amplified, purified, and digested with SacI and BglII to remove a 715-bp portion of the mdr/AFaP insert. This subfragment was replaced with a similar 715-bp fragment containing the mutation G to T at nucleotide 692 of the coding sequence. This is the mutation that was found in samples from the MSPP-1 cells. The mutated 715-bp fragment was generated as follows. RT-PCR with RNA from MSPP-1 cells was used to generate a 788-bp fragment of the mdr1 gene encompassing nucleotides 469–1256 of the coding sequence. This was carried out with the primers CAGGAGATAATCTGTTGGATGGTG and CCCTCCATCTTCTAGATTCGAG and CCCTCCATCTTCTGACTCGTGTTTG. The resulting 747-bp fragment was digested with SacI and BglII to generate the appropriate 715-bp fragment of the mdr1 gene to ligate into the opened Lit39-mdr/AFaP vector. The mdr1 portion of the recombinant Lit39-mdr/AFaP vector was sequenced to verify that no mutations other than the G to T at nucleotide 692 had been introduced. The recombinant Lit39-mdr/AFaP vector was digested with A/JII and ApoI to release the entire 1606-bp mdr1 gene, and the resulting plasmid was designated as pMTmdr vector. The resulting vector was designated as pMTmdr-692.

Purified pMTmdr and pMTmdr-692 plasmids were linearized by digestion with the enzyme AhdI and used to transfect the human HEK 293T cell line by the calcium phosphate precipitation method. HEK 293T cells exhibiting increased drug resistance were isolated by exposing the cells to 6 μM puromycin. A colony of resistant cells obtained using the pMTmdr plasmid was isolated and designated as 293Twt-3. A series of drug-resistant variants obtained using the pMTmdr plasmid was isolated and designated as 293T-801-3. These cells have increased resistance to puromycin and vincristine but not to actinomycin D. RNA samples from HEK 293T, 293Twt-3, and 293T-801-3 cells were evaluated for evidence of murine mdr1 expression using RT-PCR to generate amplified cDNA fragments (42). Only the transfectant cell lines had the expression of the mdr1 gene, and sequence analysis confirmed the expression of the normal and mutated genes in the 293Twt-3 and 293T-801-3 cells, respectively.

**Intracellular Steroid Accumulation—**Measurements of steroid accumulation in intact cells were carried out by a method outlined elsewhere (2). Briefly, lymphoid cells were collected by centrifugation and resuspended (1 × 10⁶ cells/ml) in fresh medium. ³²P-Labeled dexamethasone was added (2 × 10⁻⁶ M final concentration), and the samples were incubated for 60 min at 37 °C in a CO₂ incubator. After the incubation period, the cells were washed free of unbound hormone using cold (0 °C) phosphate-buffered saline. Retained hormone was measured with a scintillation counter.
RESULTS

Steroids can serve as substrates for Pgp efflux and/or inhibitors of drug transport (4). Previous studies demonstrated that the hydrophobicity of steroids is a strong determinant in their ability to inhibit drug transport (7, 8). We have chosen to look further into this issue using a circumscribed set of steroids, a series of 5\(b\)-pregnane compounds containing a progressively increasing number of oxygen atoms. This class of molecules was chosen because its members do not contain a double bond in the steroid A-ring, a property that prevents them from binding to glucocorticoid receptors with a significant affinity. Consequently, 5\(b\)-pregnanes do not serve as activators of the glucocorticoid receptor, an important consideration when using a cell line (MS23) that undergoes apoptosis in response to the hormone. Fig. 1 illustrates the results of an experiment comparing the ability of four pregnane compounds to reverse puromycin resistance in the MS23 cell line. MS23 cells express the \(\text{mdr}1\) Pgp (1) and are more resistant to puromycin (LC\(_{50}\); 9.0 \(\mu\)M) compared with the Pgp-deficient parental line W7TB (LC\(_{50}\); 1.5 \(\mu\)M). In this experiment, all of the MS23 cultures were grown in 3 \(\mu\)M puromycin, and the concentration of the pregnane compounds was varied. 5\(b\)-Pregnane, the most hydrophobic of the series, had no significant effect on the growth of the cells up to a concentration of 20 \(\mu\)M. 5\(b\)-Pregnane-3-one was more effective (EC\(_{50}\); 10 \(\mu\)M), followed by 5\(b\)-pregnane-3,20-dione (EC\(_{50}\); 3 \(\mu\)M). 5\(b\)-Pregnane-17\(a\)-ol-3,20-dione (EC\(_{50}\); 0.8 \(\mu\)M) was the most effective at reversing puromycin resistance. Thus, for this series of compounds, the increased potential of the steroid to make hydrogen bonding interactions enhanced the ability to block the transporter. We have observed a similar pattern for these compounds in their ability to increase accumulation of dexamethasone and daunomycin in mdr1-expressing cell lines (data not shown). These results, along with

### TABLE I

| Primer | Sequence |
|--------|----------|
| A fragment (1023 bp) | ACATCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A1 | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A1L | TACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A subfragments | |
| A1 (251 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A2 (300 bp) | GACTCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A2L | CTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A3 (301 bp) | AGCTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A3L | CAGCTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A4 (244 bp) | GACTCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A4L | CTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| B fragment (1054 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| B1 (326 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| B2 (377 bp) | GACTCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| B3 (289 bp) | AGCTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| B4 (186 bp) | GACTCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| C fragment (933 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| C1 (324 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| C2 (284 bp) | AGCTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| C3 (293 bp) | GACTCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| D fragment (1114 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| D1 (362 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| D2 (407 bp) | GACTCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| D3 (359 bp) | GACTCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| D4 (359 bp) | GACTCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| E fragment (359 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| E1 | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |

**TABLE II**

| Primer | Sequence |
|--------|----------|
| A fragment (1023 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A1 | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A1L | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A subfragments | |
| A1 (251 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A2 (300 bp) | GACTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A2L | GACTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A3 (301 bp) | AGCTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A3L | AGCTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A4 (244 bp) | GACTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| A4L | GACTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| B fragment (1054 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| B1 (326 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| B2 (377 bp) | GACTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| B3 (289 bp) | AGCTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| B4 (186 bp) | GACTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| C fragment (933 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| C1 (324 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| C2 (284 bp) | AGCTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| C3 (293 bp) | GACTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| D fragment (1114 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| D1 (362 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| D2 (407 bp) | GACTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| D3 (359 bp) | GACTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| D4 (359 bp) | GACTACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| E fragment (359 bp) | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
| E1 | CATACCGAAAGGAAAGGAAAGAGGACCTGCAGT |
Steroid Interactions with P-glycoproteins

In an effort to isolate variants expressing mutated Pgp, a sample of mutagenized MS23 cells was exposed to the combination of puromycin 5βPodo for 21 days. A single resistant clone of cells was obtained and designated as MSPP-1. The growth of this variant represents a frequency of survival of $5 \times 10^{-7}$. Fig. 3 depicts the drug resistance profile of MSPP-1 cells compared with W7TB and MS23 cells. Fig. 3A demonstrates that there was a complete reversal of dexamethasone resistance in the MSPP-1 cells. In comparison, there was no change in the vincristine resistance relative to the MS23 cells (Fig. 3B), nor was there a change in taxol resistance (data not shown). The MSPP-1 cells also exhibited increased resistance to colchicine (Fig. 3C) and a decreased resistance to puromycin (Fig. 3D) and daunomycin (data not shown). The decrease in puromycin resistance was not severe enough to compromise survival in 3 μM puromycin, the concentration used in the selection of the MSPP-1 cells.

The reversal of dexamethasone resistance in the MSPP-1 variant indicates that the Pgp expressed in these cells has lost the capacity to cause an efflux of the steroid. Therefore, MSPP-1 cells should be able to accumulate more of the hormone than the MS23 cells. Fig. 4 demonstrates that the reduction in dexamethasone accumulation, seen for MS23, was completely reversed. MSPP-1 cells accumulate as much hormone as the Pgp-deficient W7TB cells. Furthermore, if this loss of resistance and increase in hormone accumulation is due to the Pgp’s decreased capacity to bind steroids, this diminished interaction should be reflected in a loss in pregnane inhibitor activity. The fact that the vincristine resistance in MSPP-1 remained the same as MS23 has allowed a comparison of the relative effectiveness of the pregnane inhibitors in the two cell lines. This was accomplished using a strategy similar to the one used for the experiment represented in Fig. 1. A series of cultures was grown in medium containing a nontoxic (for MS23 and MSPP-1) concentration of vincristine and a range of preg-
nane concentrations. Fig. 5A shows that there was no difference in the ability of 5β-pregnane-3-one to reverse vincristine resistance between MS23 and MSPP-1 cells. In comparison, MSPP-1 required 9 times as much 5β-pregnane 3,20-dione as MS23 to reverse the resistance (Fig. 5B). We interpret the results of Fig. 5, A and B, to indicate that the putative mutation in the MSPP-1 Pgp does not have the capacity to significantly affect recognition of the 5β-pregnane-3-one portion of the structure but does alter the recognition of a 20-keto group. The results shown in Fig. 5C demonstrate that the shift in relative inhibitory capacity was even greater with 5β-pregnane-17α-ol-3,20-dione. In this instance, it required 33-fold more of the pregnane to inhibit vincristine resistance in the MSPP-1 cells. This observation indicates that the putative mutation alters the recognition of both the 20-keto and 17-hydroxyl groups and confirms the basis for survival of the MSPP-1 cells during the selection. The possibility that recognition of the 17-hydroxyl group has been compromised is supported by the results in Fig. 5D. MSPP-1 required 3-fold more 5β-androstan-17α-ol-3-one to reverse the vincristine resistance than did the MS23 cells.

Two additional selections have been made with MS23 cells and a series of variants resistant to the combination of puromycin and 5βPodo isolated. This represents frequencies of survival of 7.8 × 10⁻⁶ and 2.5 × 10⁻⁶. As in the first example, the selections were carried out in multiwell dishes with each well initially containing 10⁶ cells. This condition acts to ensure that there is a clonal isolation of variants. However, each set has the possibility of containing siblings of common progenitors due to growth during the recovery period after mutagenesis.

**Fig. 3.** Comparison of drug sensitivity profiles in W7TB, MS23, and MSPP-1 cells. Cultures of W7TB MS23 and MSPP-1 cells (5 × 10⁴ cells) were grown in increasing concentrations of the indicated drugs for 7 days. At the end of the incubation period, the turbidities of the cultures were measured and analyzed as in Fig. 1. A, dexamethasone; B, vincristine; C, colchicine; D, puromycin. ○, W7TB; ●, MS23; □, MSPP-1.

**Fig. 4.** Accumulation of dexamethasone in W7TB, MS23, and MSPP-1 cells. Samples (2 × 10⁷) of W7TB, MS23, and MSPP-1 cells were incubated with [³H]dexamethasone (2 × 10⁻⁶ M) for 1 h. At the end of the incubation, the cells were pelleted and washed (three times) to remove unbound hormone, and the amount of intracellular hormone was measured in a scintillation counter. The values of bound hormone are normalized per 2 × 10⁷ cells. Triplicate samples were analyzed.
RT-PCR was employed to generate a series of overlapping cDNA fragments spanning the entire coding sequence of the \textit{mdr1} gene expressed in a series (8) of variants and MS23 cells. Each DNA fragment was sequenced and compared with the sequence of the murine \textit{mdr1} gene (44). In each case, only one sequence difference was found between MS23 and the variant within the entire sequence of the gene. Fig. 6 depicts a side by side comparison of the MS23 and MSPP-17 sequences near the site of the mutation in the variant cell line. The analysis was of the noncoding strand. There was a complete disappearance of the C band in the MSPP-17 sample at position 901 and the generation of a band at the T lane. Thus, the difference was a G to A transition at nucleotide 901 of the coding sequence that resulted in an amino acid change of A301T. This site is within the proposed transmembrane domain 5, 6 amino acids away from the membrane/cytoplasm boundary. It is important to note that, since the DNA fragments were not cloned prior to sequencing, this change is indicative of the entire population of PCR-generated fragments. Thus, the results confirm that the expression of the \textit{mdr1} Pgp in these cell lines is from a single copy of the gene. The data also provide confirmation of the clonal nature of the variant cell lines. A mixture of expressed P-glycoprotein genes with mutations at different positions would not show a complete change from one nucleotide to another.

Table II provides a summary of the drug resistance profiles of the eight variants for six different toxic drugs including dexamethasone. Since we have chosen to study variants with a pronounced loss of steroid resistance, the results with dexamethasone reflect this precondition. None of the analyzed variants exhibited an increase in actinomycin D resistance that would be indicative of expression of the \textit{mdr3} gene. In nearly all of the variants, there was at least a 33% loss in puromycin resistance. Also listed are the five amino acid changes predicted from the mutations that were found. The results show that the W231L change was found in variants from three independent selections, while the A301V change was observed in two independent selections. The alanine 301 was found to be mutated in two ways, A301T and A301V, by changes at two adjacent bases. There is consistency in the phenotypes of the variants with the same mutation that were isolated from independent selections. More importantly, the drug resistance pro-
pressing the mutated protein required 4 times as much steroid (EC50 = 24.9 μM). Taken together, the results of Figs. 7 and 8 confirm that the mutation producing the W231L change caused a reduction in the ability of 5βPodo to inhibit drug resistance.

**DISCUSSION**

The efficiency of our strategy for obtaining variant cell lines expressing mutated Pgp is enhanced by three elements. First, the mdr1 gene in MS23 cells is expressed from a single allele, thus allowing an unambiguous evaluation of phenotypic changes resulting from the mutations. Second, the pregnant inhibitors are nontoxic steroids, structurally related to dexamethasone. Changes in the Pgp’s ability to interact with the steroid inhibitors are reflected by a decrease in dexamethasone resistance. This property allows a simple screen of a large number of variants for candidates expressing an altered Pgp function that is related to steroid recognition. Third, steroids are small molecules with a relatively limited number of sites that can contribute to interactions with the protein. Modification of one or two such interactions should have a large effect compared with other inhibitors that have a more complex interaction with the protein. To date, we have carried out four independent selections with the combinations of 5βPodo and puromycin. 67 variant cell lines were isolated. 36 of these exhibit loss of dexamethasone resistance and are currently under evaluation. A significant number of these contain the W231L mutation, suggesting that it may represent a mutational “hot spot.” We have screened the 31 variants that do not exhibit a change in steroid resistance and found a characteristic increase in resistance to actinomycin D. This property is indicative of mdr3 Pgp expression (45). Co-expression of the mdr3 Pgp, since, compared with mdr1, it is only weakly inhibited by 5βPodo (54), could convey puromycin resistance and account for survival in the selection process. We have used RT-PCR to verify mdr3 expression in several of these variant cell lines. It should be noted that this selection should not be compromised by survival of cells with glucocorticoid receptor mutations. Instead, the selection demands a retained capacity to transport puromycin, while the effectiveness of steroid Pgp inhibition is reduced. Thus, mutations resulting in a large reduction of mdr1 P-glycoprotein function will not convey a selective advantage.

The results of our analysis provide definitive support for the proposal that the mdr1 P-glycoprotein can transport dexamethasone out of cells, thus reducing the potential for activation of glucocorticoid receptors. The location of the five mutations described here is evidence that the interaction between the steroid and the Pgp involves the transmembrane portion of the first half of the protein. The decrease in 5βPodo’s inhibitory activity, which results from the mutations, suggests that the primary effect of the mutations may be upon steroid binding. Specifically, the data indicate a lost recognition of the 20-keto oxygen and 17-hydroxyl group. Fig. 1 demonstrated that both contribute to 5βPodo’s inhibitory activity. That recognition of both groups could be simultaneously affected is not surprising, since they are associated with adjacent carbon atoms. Previous studies had indicated that the 17-hydroxyl group contributed to dexamethasone’s ability to be transported (1). We believe that these observations are consistent with the keto oxygen and 17-hydroxyl group acting to promote higher affinity binding between steroids and Pgp. However, it is unlikely that 5βPodo is transported by the Pgp, since it lacks an 11β-hydroxyl group that is a key determinant for steroid transport. Thus, it appears that 5βPodo has the necessary structural elements for avid binding to the Pgp without its efficient removal from the protein through the transport mechanism. Such a combination could account for its effectiveness as a Pgp inhibitor relative to

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**Fig. 6. A comparison of the B1 fragment sequences in samples from MS23 and MSPF-17 cells.** Purified B1 fragments obtained by RT-PCR were sequenced using a 5′-32P-labeled primer. The samples were analyzed by electrophoresis through a 6% polyacrylamide urea gel, and the resulting gel was subjected to autoradiography. A portion (~1%) of the resulting pattern is depicted. The left-hand member of each pair of sequencing bands is from the MS23 sample. The right-hand member of the pair is from the MSPP-17 sample. The arrow indicates the site of a mutation in the sample from the MSPP-17 cells.
other steroids, such as dexamethasone, which are efficiently transported (3, 4).

Kajiji et al. (10) have studied the effects of a S941F mutation (transmembrane domain 11) on inhibitor activity in Chinese hamster ovary cells transfected with the \textit{mdr}1 gene. They reported that this mutation caused a strong reduction of pho- 
toaffinity labeling by iodoarylazidoprazosin and azidopine along with reduced vinblastine, adriamycin, and colchicine re- 
sistance. Compared with the normal Pgp, progesterone was shown to cause a smaller reversal in resistance to these three 
 drugs with the mutated protein. However, this reversal was an 
inhibition of an already compromised resistance, and the ef- 
facts that they observed were the result of exposure to a single 
concentration (25 \text{ m}\text{M}) of progesterone. In our studies (Fig. 5), 
we used a titrated reversal of vincristine resistance to evaluate 
changes in the efficacy of the pregnane inhibitors, specifically 
because the vincristine sensitivity did not differ between MS23 
and the variant MSPP-1. In this way, we were able to deter- 
mine the relative concentrations of pregnanes needed to cause 
an equivalent reversal of drug resistance in the two cell lines.

Others have used the combination of a Pgp inhibitor and 
toxic drug in an effort to isolate variants expressing Pgp mu-
tations affecting inhibitor activity. Chen et al. (14) reported the 
isolation of a human sarcoma line (DxP) expressing a mutated 
\textit{MDR1} Pgp that exhibited a decreased response to cyclosporin- 
based transport inhibitors. The DxP cell line was isolated 
through a prolonged (1-year) selection in the presence of the 
PSC-833 inhibitor and increasing concentrations of doxorubicin 
(40–500 nM). The DxP cells displayed decreased resistance to 
daunorubicin, paclitaxel, Vinca alkaloids, and epipodophylla-

\begin{table}[h]
\centering
\caption{Profiles of changes in drug sensitivities for variants expressing mutated \textit{P-glycoproteins}}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Cell line}\textsuperscript{a} & \textbf{VINC} & \textbf{DNR} & \textbf{DEX} & \textbf{PURO} & \textbf{COLC} & \textbf{ACT D} & \textbf{Mutation} \\
\hline
MSPP-1 (1) & NC & ↓ & ↓ & ↓ & ↓ & ↓ & ++ & NC W231L \\
MSPP-7 (2) & NC & ↓ & ↓ & ↓ & ↓ & ↓ & ++ & NC W231L \\
MSPP-55 (3) & NC & ↓ & ↓ & ↓ & ↓ & ↓ & ++ & NC W231L \\
MSPP-6 (2) & + & NC & ↓ & ↓ & ↓ & ↓ & NC H346N \\
MSPP-17 (2) & ↓ & ↓ & ↓ & ↓ & ↓ & ↓ & NC A301T \\
MSPP-4 (2) & NC & NC & ↓ & ↓ & ↓ & ↓ & NC A301V \\
MSPP-35 (3) & NC & NC & ↓ & ↓ & NC & ↓ & NC A301V \\
MSPP-21 (2) & ↓ & ↓ & ↓ & ↓ & ↓ & ↓ & NC A301V \\
\hline
\end{tabular}
\textsuperscript{a} The numbers within the parentheses refer to the selection experiment in which the variant was isolated. 
\textsuperscript{b} VINC, vincristine; DNR, daunorubicin; DEX, dexamethasone; PURO, puromycin; COLC, colchicine; ACT D, actinomycin D. 
\textsuperscript{c} NC, no change.
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{The locations of pregnane-derived P-glycoprotein mutations within the proposed relationship between the protein and the plasma membrane. The filled circles represent the amino acids that are altered by the mutations found in the MSPP variants.}
\end{figure}
toxins but not doxorubicin. DxD also manifested decreased capacity to transport cyclosporin and rhodamine 123. In comparison with the point mutations that we have observed, the mutation in the DxD MDR1 gene was a deletion that resulted in the loss of the Phe335 amino acid in the transmembrane domain 6 portion of the protein. Site-directed mutagenesis has also been used to explore the effects of substitution mutations at the Phe335 position (19). A F335A or F335S change caused increased resistance to doxorubicin. A F335L change caused decreased resistance to doxorubicin. The effects of these changes on the activity of cyclosporin inhibitors was not reported.

Photoaffinity labeling experiments have indicated that there are two regions of the Pgp that are involved in drug binding (46–51). These regions include the transmembrane domains 5 and 6, as well as the 11 and 12 domains. More recently, Demmer et al. (52) used iodomycin, a modified form of daunomycin, to map an anthracycline binding site that included the 4 and 5 transmembrane domains of a hamster Pgp. Our results are in accordance with that observation. Three of the four steroid-related point mutations that we have found, located in transmembrane domains 4 and 5, cause a decrease in daunomycin resistance (Table II). Using a similar approach, Wu et al. employed two benzophenone analogues of taxol to photoaffinity label the mouse mdr1b (mdr1) Pgp (53). Each compound preferentially labeled a different region located in the second half of the protein. One labeled a fragment that includes half of transmembrane domain 12 and extends past the Walker A motif in the second ATP binding region. The other labeled a region that encompassed all of transmembrane domain 7 and half of 8. Taken together, the results of these studies suggest the possibility of overlapping steroid-anthraclycline sites within the first half of the protein and another taxane site within the second half of the protein.

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