0026-895X/01/6001-104–113$3.00
MOLECULAR PHARMACOLOGY
Vol. 60, No. 1
704/908672
Mol Pharmacol 60:104–113, 2001
Printed in U.S.A.

The Use of a Novel Taxane-Based P-Glycoprotein Inhibitor to Identify Mutations That Alter the Interaction of the Protein with Paclitaxel

DONALD J. GRUOL, JOSH BERND, ALBA E. PHIPPARD, IWAO OJIMA, and RALPH J. BERNACKI

The Sidney Kimmel Cancer Center, San Diego, California (D.J.G, J.B., A.E.P.); Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York (I.O.); and Department of Pharmacology and Therapeutics, Grace Cancer Drug Center, Roswell Park Cancer Institute, Buffalo, New York (R.J.B)

Received November 11, 2000; accepted March 14, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Murine thymoma cell lines expressing mutated forms of the *mdr*1b P-glycoprotein were isolated using a novel taxane-based P-glycoprotein inhibitor tRA-96023 (SB-RA-31012). The selection strategy required resistance to a combination of tRA-96023 and colchicine. Five mutations were identified (N350I, I862F, L865F, L868W, and A933T) that reduce the capacity of tRA-96023 to inhibit P-glycoprotein-dependent drug resistance. These mutations also result in a loss of paclitaxel resistance ranging from 47 to 100%. Four mutations are located in the second half of the protein, within or near the proposed transmembrane segment (TMS) 10–11 regions. The fifth mutation (N350I) is within the first half of the protein, proximal (cytoplasmic) to TMS 6. The variant cell line expressing the L868W mutation was subjected to a second round of selection involving tRA-96023 and the toxic drug puromycin. This resulted in the isolation of a cell line expressing a P-glycoprotein with a double mutation. The additional mutation (N988D) is located within TMS 12 and conveys further decreases in resistance to paclitaxel and the capacity of tRA-96023 to inhibit drug resistance. Taken together, the results indicate a significant contribution by the TMS 10–12 portion of the protein to the recognition and transport of taxanes and give evidence that the cytoplasmic region proximal to TMS 6 also plays a role in taxane interactions with P-glycoproteins. Interestingly, mutations within TMS 6 and 12 were found to cause a partial loss of PSC-833 inhibitor activity, suggesting that these regions participate in the interactions with cyclosporin and its derivatives.

P-glycoprotein expression conveys multidrug resistance through the ability of the protein to interact with hydrophobic drugs and to cause their efflux out of cells (Kane, 1996; Bradshaw and Arceci, 1998; Ambudkar et al., 1999). This behavior is likely to involve a capacity to intercept hydrophobic compounds as they diffuse across/within the plasma membrane (Shapiro et al., 1997; Eytan and Kuchel, 1999) and to transfer them into the external aqueous milieu. The prevailing model of P-glycoprotein structure predicts 12 TMS, six within each half of the protein. These sections make up approximately 20% of the molecule. Electron microscopic studies have indicated that the protein has an overall toroidal shape, closed in the center at the cytoplasmic interface, with a central aqueous region facing the external environment (Rosenberg et al., 1997). The relationship of the 12 TMS to the rest of the structure has not been defined, but Loo and Clarke (1999, 2000), using cysteine-scanning mutagenesis and thiol-modification techniques, found evidence that the TMS 4 to 6 interact with TMS 12, whereas TMS 10 to 12 interact with TMS 6. The exit pathway for the transported drugs is also not well delineated. Substrates may be "flipped" from the inner to the outer membrane leaflet (Higgins and Gottesman, 1992), moved into an intermediate pore-like structure before subsequent release (Ashida et al., 1998), or transferred directly out into the aqueous compartment. The latter two scenarios represent versions of what has been termed the "vacuum cleaner" model (Raviv et al., 1990).

The TMS 4 to 6 and TMS 10 to 12 regions have been implicated in the binding of drugs. Much of the evidence for this view was developed through photoaffinity labeling of the protein (Bruggemann et al., 1989, 1992; Greenberger et al., 1990, 1993; Morris et al., 1995; Zhang et al., 1995). More recently, a derivative of daunomycin was shown to selectively cross-link with a peptide from the first half of a hamster P-glycoprotein spanning the inner part of TMS 4, the second cytoplasmic loop, and the inner part of the TMS 5 region (Demmer et al., 1997). Similarly, photoactive derivatives of paclitaxel implicated the TMS 7 to 8 and 11 to 12 in the binding of taxanes to the second half of the murine mdr1b

This work was supported by National Institutes of Health Grants DK51107 (D.J.G.), GM42798 (I.O.), and CA73872 (R.J.B.)

ABBREVIATIONS: TMS, transmembrane segment; 5βPodo, 5β-pregnane-17ol-3,20-dione; PCR, polymerase chain reaction; Pgp, P-glycoprotein.

protein (Wu et al., 1998). Thus, depending upon the drug, there may be separate sites on each half of the protein or sites composed of TMS from both halves of the protein. This type of combinatorial diversity could help to explain the capacity of these proteins to transport such a large number of unrelated compounds.

The broad range of compounds that function as P-glycoprotein substrates and/or inhibitors also raises the question if there are distinct individual binding sites or binding zones that provide overlapping recognition of a spectrum of unrelated molecules. The physical relationship between any one binding site to another remains unknown. A wide variety of mutations, often obtained by site-directed mutagenesis (Currier et al., 1989; Dhir et al., 1993; Kajiji et al., 1993; Loo and Clarke, 1993; Hanna et al., 1996; Loo and Clarke, 1996; Bakos et al., 1997; Loo and Clarke, 1997; Taguchi et al., 1997; Hafkemeyer et al., 1998; Kwan and Gros, 1998), have been shown to alter the "specificity" of P-glycoprotein-dependent drug resistance profiles. That is, depending upon the given mutation, the individual resistances to a set of drugs can be altered in a variety of different ways involving increases, decreases, and no change. Moreover, changing a single amino acid to alternative forms can also produce a spectrum of drug resistance profiles (Loo and Clarke, 1993). This variable behavior and the dispersed location of the mutations has made it difficult to conclude that specific drugs interact with defined portions of P-glycoproteins. Part of this difficulty is likely to reside in the fact that resistance/drug transport is dependent not only upon drug binding, but also upon activation of ATPase activity and translocation of the drug out of the membrane. Mutations affecting any one of these steps could differentially alter the transport of a subset of drugs, thus introducing changes in the specificity. In an attempt to focus upon the drug binding step, efforts have been made to identify mutations that reflect an altered capacity of inhibitors to block transport. Chen et al. (1997) used a combination of doxorubicin and the inhibitor PSC 833 to isolate a human sarcoma line (Dxp) expressing a mutated human MDR1 P-glycoprotein. The mutation was a deletion that resulted in the loss of Phe335, which is located in the TMS 6 portion of the protein. Vo and Gruol (1999) also employed the combination of inhibitor and toxic drug to isolate murine thymoma cell lines expressing mutated P-glycoproteins. In this instance, the combination of a steroid inhibitor, 5β-pregnane-17α-ol-3,20-dione (5βPodo), was used with puromycin to identify five point mutations. These mutations resulted in a loss of dexamethasone resistance and increased steroid accumulation in the variant cell lines. All five of the mutations are clustered within the inner leaflet portion of the TMS 4 to 6 region of the protein, thus, providing evidence that steroids initially interact with the first half of the murine mdr1b Pgp. The results presented below describe an extension of the combination selection approach to ask which portion(s) of the murine mdr1b protein interacts with taxanes.

Materials and Methods

Cell Culture. MDA/LCC6 is a human breast carcinoma line and LCC6-MDR is a variant that expresses a transfected hMDR1 P-glycoprotein gene. Both lines were generously provided by Dr. R. Clarke, Lombardi Cancer Center, Georgetown University School of Medicine (Washington, DC). These two cell lines were propagated as monolayers in RPMI-1640 medium containing 5% fetal bovine serum, 5% NuSerum IV, 20 mM HEPES, and 2 mM L-glutamine. The incubator was maintained at 5% CO_2 and 95% air.

WEHI-7 is a thymoma cell line obtained from a female BALB/c mouse after exposure to X-irradiation (Harris et al., 1973). It is sensitive to killing by corticosteroids. The W7TB cell line is a derivative of WEHI-7 that is resistant to bromodeoxyuridine. Bromodeoxyuridine resistance is unrelated to steroid or multidrug resistance. MS23 is a variant of W7TB selected through prolonged growth in low levels of dexamethasone and expresses the murine mdr1b P-glycoprotein (Bourgeois et al., 1993) from a single copy of the gene (Vo and Gruol, 1999). All the murine 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% CO_2 and 87% air.

Selection of MS23 Variants. Independent selections were initiated by the mutagenesis of MS23 cells with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, obtained from Sigma-Aldrich (St. Louis, MO). This compound is an alkylating agent that predominantly causes point mutations. After allowing sufficient time for the cells to recover and express the mutated genes (usually 4–5 days), the cells were dispensed into multiwell dishes (1×10^5 cells/well) in medium containing a toxic drug and the taxane-based P-glycoprotein inhibitor tRA-96023 (Ojima et al., 1998). Three independent mutagenesis/selection experiments have been carried out. In the first two, the MSTC-n variants were isolated from MS23 cells in 40 ng/ml colchicine and 250 nM tRA-96023. In the third selection, variants were isolated from the MSTC-24 variant using the combination of 5 μM puromycin and 1.5 μM tRA-96023. Resistant colonies typically appeared between 12 and 18 days. Each of the variant lines was initially screened for changes in growth in the presence of paclitaxel relative to MS23 cells or MSTC-24 cells. The results of these evaluations identified those variants that had lost significant paclitaxel resistance.

Quantification of Drug Effects on Cellular Proliferation. Assessment of cell growth inhibition in the human MDA-435/LCC6 cells was determined according to the methods of Skehan et al. (1990). Paclitaxel, doxorubicin, vinblastine, and the taxane reversing agent tRA-96023 were solubilized in DMSO and further diluted in RPMI-1640 medium containing 10 mM HEPES. Each cell line was treated with 10 concentrations of the toxic drug (5 log range) $\pm$ tRA-96023. After a 72-h incubation, the cells were fixed with trichloroacetic acid and stained with a 0.4% solution of sulforhodamine B. Absorbance was measured at 570 nm. IC_{50} values were calculated relative to cells that were not exposed to the toxic drug.

The effect of drugs on the murine thymoma cell proliferation was measured as follows: cell cultures were set up (5×10^4 cells/ml) 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 $<5\%$ represent situations in which all of the cells have lost viability. The IC_{50} value is defined as the concentration of drug that produces a relative turbidity value of 50%.

The relative ability of nontoxic inhibitors to reverse P-glycoprotein-dependent drug resistance in cells was evaluated as follows: A series of cultures (5×10^4 cells/ml) were grown with a fixed concentration of a toxic drug to which that cell line is normally resistant based upon its P-glycoprotein expression. Increasing concentrations of the inhibitor were included in the culture medium and the relative turbidity values of the cultures evaluated after 7 days. The inhibitory efficiency of the inhibitor is expressed by an EC_{50} value defined as the concentration of chemosensitizer which reduces the relative turbidity value to 50%. The steroid 5β-pregnane-17α-ol-3,20-dione was obtained from Steraloids Inc. (Newport, RI). PSC-833 was a gift

provided by Novartis Pharmaceuticals Corp (Basel, Switzerland; formerly Sandoz).

Evaluation of *mdr*1 P-Glycoprotein Mutations Expressed in the MS23 Variants. Reverse transcription-PCR was used to generate a series of overlapping cDNA fragments encompassing the entire coding sequence of the *mdr*1 gene expressed in MS23 and the variant cell lines (Vo and Gruol, 1999). Five larger primary fragments were initially produced, and a series of subfragments were generated using a set of nested primers. The individual PCR products were purified by agarose gel electrophoresis and evaluated by direct DNA sequencing using the appropriate primers (*fmol* DNA Sequencing System; Promega, Madison, WI) that had been end-labeled with ^{32}P. All primers were obtained from Invitrogen (Carlsbad, CA).

Results

Figure 1 shows the structures of paclitaxel and a taxane-based compound, tRA-96023 (SB-RA-31012). The tRA-96023 molecule contains a core portion of paclitaxel to which a benzoyl-dihydrocinnamoyl group has been esterified at the 7-carbon atom (Ojima et al., 1998). This compound lacks the (2*R*, 3*S*)-*N*-bensoylphenylisoserine group and has no toxicity in vitro up to a solubility-limiting concentration of 30 μM (data not shown). tRA-96023 was developed as a potential inhibitor of P-glycoprotein function based upon the drug's similarity to paclitaxel, which is a substrate for the transporter. Table 1 lists the results of studies evaluating the ability of tRA-96023 to reverse drug resistance in a human breast tumor cell line (MDA 435/LCC6^{mdr}) that over-expresses the MDR1 P-glycoprotein. Three toxic drugs were employed: paclitaxel, doxorubicin, and vinblastine. In each case, the IC_{50} was determined for the parental cell line (MDA 435/LCC6^{wt}) and compared with the IC_{50} values of the P-glycoprotein-expressing line in the absence and presence of tRA-96023. Most of the P-glycoprotein-dependent paclitaxel resistance was reversed by tRA-96023, and there was a 92 and 99% reversal of resistance to doxorubicin and vinblastine, respectively. tRA-96023, by itself, had no effect on the growth of these cell lines (not shown).

The rationale for developing a taxane-derived P-glycoprotein inhibitor is based upon the concept that P-glycoproteins interact with specific structural features of a drug to produce a mechanistically relevant binding complex. This specificity should represent drug-protein interactions that promote retention/transport of the drug. A similar logic lies behind the approach taken by Wu et al. (1998), who used compounds structurally related to tRA-96023 as photoaffinity labeling probes. Their goal was to identify the region(s) of the protein that preferentially interact with taxanes. We have chosen to investigate the location of taxane P-glycoprotein interactions through a different but complementary approach: identifying mutations that alter the inhibitory activity of the tRA-96023 molecule. To accomplish this goal, we have employed a strategy that has proven to be successful at identifying sites that are important in steroid interactions with the Pgp (Vo and Gruol, 1999). The first step in the process is to isolate, based on a decreased ability to be blocked by the inhibitor, cell lines expressing mutated Pgp. This is greatly facilitated by the use of the murine thymoma cell line MS23, which expresses the mdr1b Pgp from a single copy of the gene. Therefore, when a mutated Pgp is expressed in a variant of these cells, the monoploid expression of the protein provides for unambiguous phenotypic changes, uncomplicated by the presence of normal protein. Variants of the MS23 line can be selected using a combination of an inhibitor and a toxic drug. In the initial selections described below, colchicine was employed as the toxic drug. Under these circumstances, the cells are usually killed because the P-glycoprotein cannot function. However, variants can be isolated that survive the challenge. There are several possible phenotypes, related to changes in P-glycoprotein function, that can be expected: a decreased responsiveness to the inhibitor, an increased resistance to colchicine, or a combination of both traits. Other, non–Pgp-dependent mechanisms are possible, but these can be recognized and eliminated from further consideration.

Two independent selections, involving colchicine and tRA-96023, were carried out that yielded a total of 38 variants for a combined frequency of 5×10^{-7}. These variants were subjected to a preliminary screen for changes in resistance to paclitaxel, colchicine, and actinomycin D. None exhibited increased resistance to actinomycin D, which would have signaled the onset of *mdr*1a (*mdr*3) expression. All of the variants displayed increased colchicine resistance, whereas 21 had reduced resistance to paclitaxel. Thus, it seems that increased colchicine resistance contributed to the survival of the variants. This phenomenon is not unique. We (Vo and

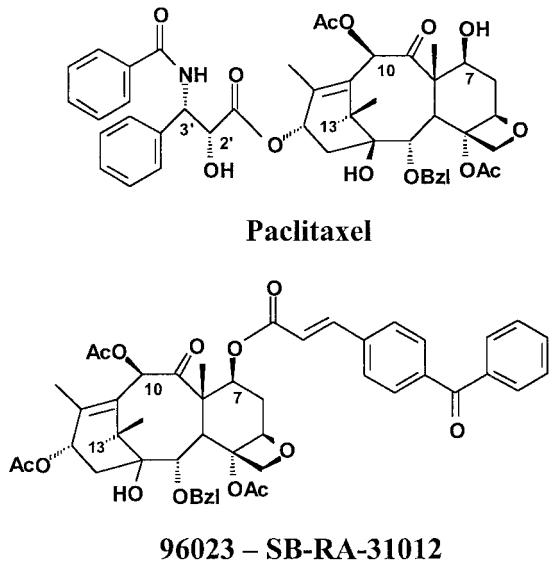

Fig. 1. Structures of paclitaxel and a taxane-based P-glycoprotein inhibitor tRA-96023 (SB-RA-31012).

TABLE 1

Modulation of sensitivity to chemotherapeutic agents by the taxane reversal agent tRA 96023

The measurements of cell sensitivities to the toxic drugs is described under *Materials and Methods.*

Condition	Cell Line[a]	IC_{50}	IC_{50} Ratio[b]
Paclitaxel	LCC6-WT	3.3 nM	
Paclitaxel	LCC6-MDR	348 nM	105
Paclitaxel + tRA 96023	LCC6-MDR	3.8 nM	1.2
Doxorubicin	LCC6-WT	0.2 μM	
Doxorubicin	LCC6-MDR	3.9 μM	20
Doxorubicin + tRA 96023	LCC6-MDR	0.5 μM	2.5
Vinblastine	LCC6-WT	1.9 nM	
Vinblastine	LCC6-MDR	86 nM	85
Vinblastine + tRA 96023	LCC6-MDR	2.5 nM	1.3

[a] MDA435/LCC6 a human breast carcinoma: WT, wild type; MDR, transfected with the human MDR1 gene.

[b] IC_{50} RATIO = IC_{50}LCC6-MDR/IC_{50}LCC6-WT.

Gruol, 1999) and other researchers (Choi et al., 1988) have identified Pgp mutations that convey increased colchicine resistance. It should be noted that, analysis of the period of recovery after mutagenesis allowed us to derive sets of variants from common progenitors. Accordingly, we have identified six different mutations in 21 (of 21) variant cell lines.

Figure 2 shows a comparison of paclitaxel sensitivities in five cell lines. They are MS23 and its parental cell line, W7TB (no P-glycoprotein expression), and three variants isolated from a selection with colchicine and tRA-96023: MSTC-8, -14, and -24. Each of the variant cell lines displays a loss in paclitaxel resistance relative to MS23. The greatest loss of resistance is evident with the MSTC-14 line, amounting to a reversal of more than 90%. RNA was isolated from MS23 along with each of the variant cell lines and subjected to reverse transcription-PCR to generate a series of overlapping DNA fragments spanning the entire coding sequence of the *mdr*1b gene. These fragments were used for direct DNA sequencing, and a single point mutation was found in the samples of each of the variant cell lines. A list of the amino acid changes that are caused by the mutations is presented as part of Table 2. Table 2 also provides a summary of the changes in drug resistance profiles for six MSTC variants of MS23. (The 24TCTP-6 line was selected by sequential selections and will be described later in the text.) All of the MSTC variants display a significant increase in colchicine resistance that undoubtedly contributed to their survival in the selections. P-glycoprotein inhibitors can reverse all of this resistance (described below). Only the mutation in the MSTC-20 cells (L338F) fails to exhibit a significant decrease in paclitaxel resistance. The three mutations in MSTC-8, -14, and -21.10 have a virtually complete loss: 84, 94, and 100%, respectively. Table 3 lists the quantitative changes in paclitaxel resistance for all seven of the variant cell lines. With the exception of the MSTC-2 cell line, none of the variants had a significant change in puromycin resistance. Three closely spaced mutations (I862F, L865F, and L868W) in the MSTC-14, -24, and -2 lines show an increased resistance to dexamethasone. All of these mutations are within the TMS 10 portion of the protein. On the other hand, the loss of dexamethasone resistance (85%) observed in the MSTC-20 cells is consistent with other mutations reported previously to be located within the first half of the protein (TMS 4–6). Figure 3 illustrates the location of the amino acid changes relative to the proposed relationship between the protein and the plasma membrane. Four of the mutations affecting paclitaxel resistance are located in the second half of the molecule, three of which reside in the transmembrane domain 10 segment. The fourth is located just within the cytoplasmic compartment, proximal to transmembrane domain 11. A fifth mutation affecting paclitaxel resistance (N350I), is located just within the proposed cytoplasmic domain, proximal to TMS 6. The sixth mutation (L338F), expressed in the MSTC-20 cell line, is located at the middle of transmembrane domain 6.

The original assumption made in selecting the variant cell lines was that the mutations would cause a loss in the recognition of tRA-96023 that would be reflected in a diminished inhibitory activity of the compound. Table 2 showed that the *mdr*1b mutations in the MSTC-8, -12, -14, -20, -21.10, and -24 lines did not significantly affect their puromycin resistance. Thus, we were able to compare the relative abilities of the inhibitors to reverse a comparable degree of puromycin resistance in these cell lines relative to MS23. The results are shown in Fig. 4. In each instance, 5 μM puromycin was added to the cultures as the concentration of one of three inhibitors was varied. This puromycin concentration is toxic to these cells when approximately 60% of the resistance is reversed. Figure 4A depicts the relative concentrations of the tRA compound needed to reverse puromycin resistance in the variants relative to MS23. The greatest shifts were seen with the MSTC-14, -21.10, and -24 cell lines where 2-fold, or more,

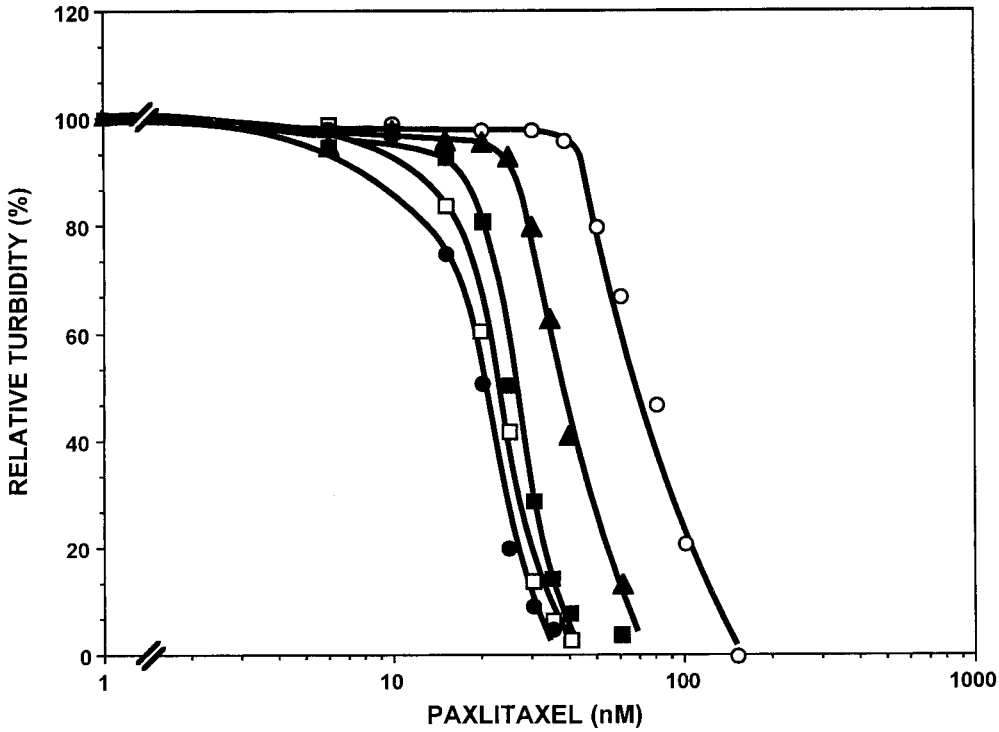

Fig. 2. Sensitivities of W7TB, MS23 and three variant cell lines to paclitaxel. Cell cultures, initially containing 5×10^4 cells/ml, were incubated in medium containing the indicated concentrations of paclitaxel for 5 days. At the end of the incubation period, the turbidities (660 nm) of the cultures were measured and expressed relative to cultures containing no drug. Each point is the average of 2 determinations. □, W7TB cells; ○, MS23 cells; ■, MSTC-8 cells; ●, MSTC-14 cells; ▲, MSTC-24 cells.

of the inhibitor (compared with MS23) was needed to produce inhibition. Only the MSTC-20 cells were unaffected, inhibited at the same concentration as MS23 cells. This result indicates an unaltered recognition of tRA-96023 in the MSTC-20 cells and suggests that their increase in colchicine resistance was the sole reason for their survival during the selection of the variants. In Fig. 4B, the pregnane inhibitor 5βPodo was used. Its structure is illustrated in Fig. 4D. Five of the mutations did not cause a decrease in this inhibitor's ability to reverse drug resistance. Only the mutation found in the MSTC-20 (L338F), located in transmembrane domain 6, resulted in a significant increased concentration of inhibitor (~10-fold) being needed to reverse the puromycin resistance. This behavior is consistent with previous results indicating the existence of a steroid interaction domain within the transmembrane sections of the first half of the protein (Vo and Gruol, 1999). Figure 4C depicts the concentrations of the inhibitor PSC 833 needed to reverse puromycin resistance in the variants. The results are similar to those obtained with 5βPodo. Only the MSTC-20 cell line shows evidence of a decrease in the potency of the inhibitor. This result is consistent with previous experiments (Chen et al., 1997; Ma et al., 1997) demonstrating that mutations in the TMS 6 of the human MDR 1 and hamster Pgp1 proteins caused a decreased PSC 833 activity. Taken together, the results suggest that steroids and taxanes interact with the P-glycoprotein at separate sites and support a role for TMS 6 in the interaction with PSC 833.

All of the isolated MSTC cell lines are capable of survival and growth under the conditions (40 ng/ml of colchicine, 250 nM tRA-96023) that were used in the selections (data not shown). Moreover, as demonstrated in Table 2, the MSTC cell lines exhibit substantial increased resistance to colchicine. This phenomenon is not unique for the MSTC variants. It was also observed with the MSPP-1 line, one of the variants that were obtained from MS23 using the combination of a steroid inhibitor along with puromycin as the toxic drug (Vo and Gruol, 1999). However, because colchicine was the toxic agent used to select the MSTC lines, it is feasible that a portion of their colchicine resistance could be caused by a mechanism not involving P-glycoproteins. To evaluate this possibility, we have tested several P-glycoprotein inhibitors for their abilities to completely reverse colchicine resistance in the MSTC cell lines. Both verapamil and PSC-833 reversed colchicine resistance in these cell lines, producing IC_{50} values comparable with that of cells (W7TB) that do not express P-glycoproteins (data not shown). Figure 5 illustrates similar results using the 5βPodo inhibitor, but with an illustrative distinction. A concentration of 10 μM is sufficient to completely reverse colchicine resistance in all of the cell lines except MSTC-20, where the reversal was 81%. The incomplete reversal by the pregnane compound in the MSTC-20 cell line, however, highlights the reduced effectiveness of the steroid inhibitor compared with the L338F mutation that was depicted in Fig. 4B. At higher concentrations (20 μM) the reversal of colchicine resistance in the MSTC-20 cell line was greater than 95% (data not shown). Thus, the results strongly support the likelihood that the increase in colchicine resistance in the MSTC cell lines is caused entirely by a change in P-glycoprotein, not microtubule function.

Although most of the mutations cause a significant reduction in paclitaxel resistance, their effects upon the inhibitory activity of the tRA-96023 compound are relatively modest, particularly compared with those seen with steroid inhibitors (Fig. 4B). This behavior is not totally unexpected because tRA-96023 is a larger and more potent inhibitor that has a potentially complex interaction with the protein. A single point mutation may not be as disruptive to tRA-96023 binding compared with steroid binding. However, this situation offers an opportunity to seek second mutations that cause an additional effect upon paclitaxel resistance. For instance, the MSTC-24 cell line expresses a Pgp mutation (L868W) that conveys a partial (55%) loss of paclitaxel resistance and a 2.4-fold increase in the dose (EC_{50}) of tRA-96023 needed to reverse drug resistance. The strategy of paired drug selection can be reiterated with this cell line using a combination of the tRA-96023 and a different drug, such as puromycin. One such selection was carried out and resulted in the identification of variants expressing the double mutation L868W, N988D. The location of the second mutation is within the TMS 12. The changes in the drug resistance profiles of one of these variants are illustrated in Table 2 for the 24TCTP-6 cell line. There were four significant phenotypic changes that resulted from the second mutation: 1) there was a change in paclitaxel resistance from −55% for MSTC-24 to −98% for 24TCTP-6; 2) the increase in colchicine resistance (220%) that was gained through the first mutation was completely reversed as a result of the second mutation; 3) there was a coincident increase in both dexamethasone and puromycin resistance (IC_{50} > twice that of MS23 cells); 4) there was a further decrease in the effectiveness of the tRA-96023 inhibitor. The latter property is illustrated in Fig. 6. This experiment takes advantage of the fact that there was no significant change in daunomycin resistance between MSTC-24 and 24TCTP-6. Therefore, a constant concentration of dauno-

TABLE 2

Summaries of changes in the drug resistance profiles of the MSTC variants

Changes in drug resistance were evaluated by measuring the IC_{50} values for the individual drugs in the W7TB, MS23, and variant cell lines. The change in drug resistance was calculated according to the equation: Change = $({}^{Var}IC_{50} - {}^{MS23}IC_{50})/({}^{MS23}IC_{50} - {}^{W7TB}IC_{50})$, where Change ≥0.5 = +, ≥1.0 = ++, ≥1.5 = +++, ≥2.0 ++++, ≤−0.33 = ▼, ≤−0.67 = ▼▼, and ≤−1.0 = ▼▼▼. NC indicates no change, or changes smaller than those indicated above, in the drug resistance.

Cell Line	Paclitaxel	Vincristine	Daunomycin	Dexamethasone	Puromycin	Colchicine	Mutation
MSTC-2	▼	NC	++	++	+	++++	L865F
MSTC-8	▼▼	▼	NC	NC	NC	+++	A933T
MSTC-14	▼▼	▼	NC	++	NC	++++	I862F
MSTC-20	NC	NC	NC	▼▼	NC	++++++	L338F
MSTC-21.10	▼▼▼	▼	+	NC	NC	++++++	N350I
MSTC-24	▼	▼	NC	+	NC	++++	L868W
24TCTP-6	▼▼	▼	NC	++++++	++	NC	L868W N988D

mycin could be employed to test the relative effectiveness of tRA-96023 in the two cell lines. In this instance, the EC_{50} values have been normalized to that of MSTC-24. The figure illustrates that nearly twice the amount of tRA-96023 was needed to reverse daunomycin resistance in the 24TCTP-6 cells compared with MSTC-24. This behavior is indicative of the N988D mutation causing a further loss in the ability of the tRA-96023 compound to interact productively with the protein. In contrast, when the 5βPodo inhibitor was used, the concentration needed to reverse resistance in 24TCTP-6 decreased by 50%. This observation suggests an increased capacity of the steroid to interact with the protein, which is also consistent with the increased dexamethasone resistance observed for this cell line (Table 2). Figure 6 also illustrates the effect of the mutation on the PSC-833 inhibitor. The EC_{50} valued increased to more than 4-fold, indicating that the N988W mutation also interferes with this inhibitor's ability to interact with the protein. Thus, mutations in both halves of the protein (L338F, N988D) can alter the effectiveness of PSC-833.

TABLE 3

Percentage loss in paclitaxel resistance for the variant cell lines listed in Table 2

A portion of the data that was used to generate Table 2 was converted from fractional loss to percentage loss of paclitaxel resistance. The values represent the mean ± S.E. for n measurements of a given cell line.

Cell Line	Paclitaxel
MSTC-2	−47% ± 23% (n=4)
MSTC-8	−86% ± 17% (n=5)
MSTC-14	−94% ± 11% (n=5)
MSTC-20	−31% ± 20% (n=4)
MSTC-21.10	−1.00 ± 10% (n=3)
MSTC-24	−55% ± 19% (n=4)
24TCTP-6	−98% ± 16% (n=2)

Discussion

There is limited information regarding the location of a paclitaxel binding site within Pgp. Wu et al. (1998) mapped the interaction domains of 2 photoreactive paclitaxel analogs within the murine mdr1b Pgp. A C-3′ *p*-benzoyl dihydrocinnamoyl-modified analog labeled a fragment (aa 985-1088) that includes the inner half of TMS 12 and terminates after the Walker A motif in the second half of the protein. In contrast, a C-3′ *p*-benzoyl dihydrocinnamoyl-modified analog reacted with a fragment (amino acids 683–760) that includes all of TMS 7, the outer half of TMS 8 and the included extracellular loop. Thus, both probes labeled regions of the protein that are within the second half of its primary structure. Before the studies presented here, mutational analysis had been unable to provide a consistently localize the interaction of paclitaxel with the protein. To address this issue, we have employed a system described by Vo and Gruol (1999) for mapping a steroid interaction region within the TMS 4 to 6 portion of the mdr1b protein. The system involves the use of a pair of murine thymoma cell lines, W7TB and MS23, that are derivatives of the WEHI-7 line isolated by Harris et al. (1973). Both cell lines are killed by glucocorticoids and MS23 was isolated from W7TB based on increased resistance to dexamethasone. The MS23 line exhibits a multidrug resistance phenotype and expresses the *mdr*1b, but not the *mdr*1a, gene. Of the five mutations reported by Vo and Gruol to affect steroid-Pgp interactions, only one (S222T) causes a

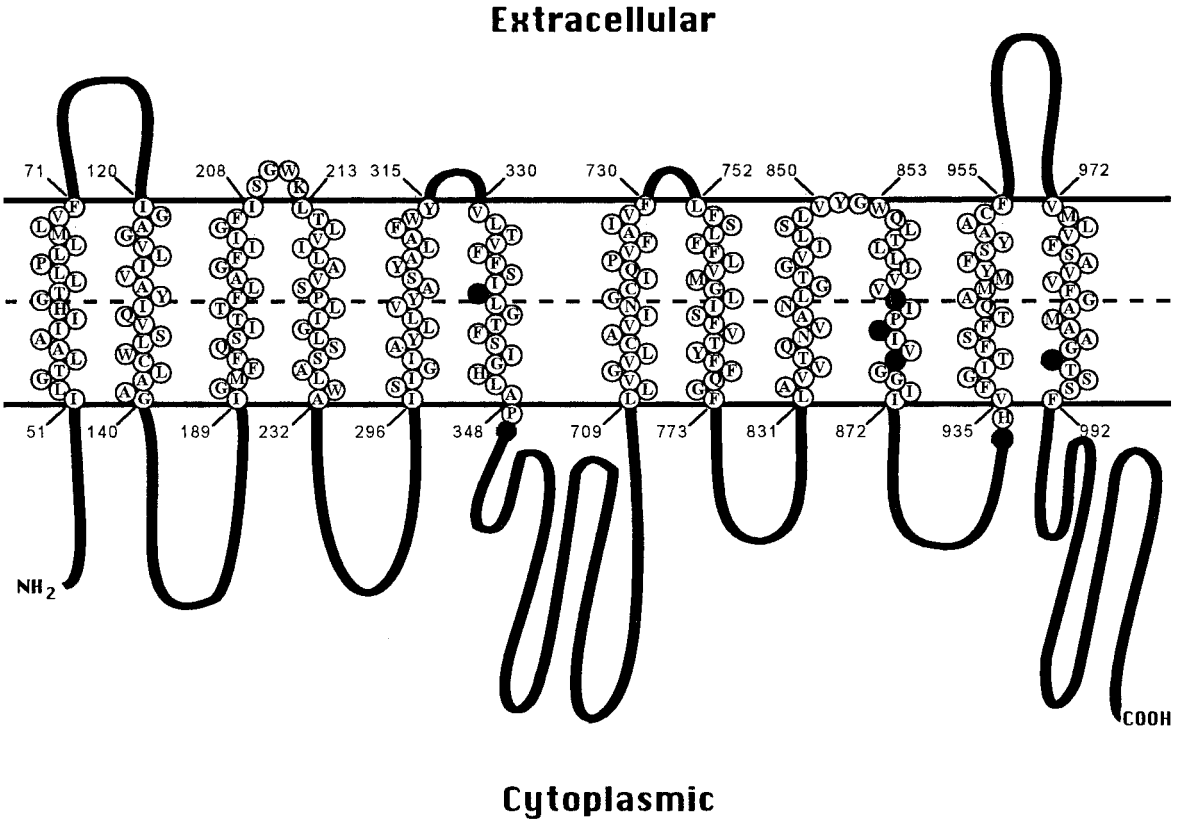

Fig. 3. The locations of taxane-derived P-glycoprotein mutations within the proposed relationship between the protein and the plasma membrane. The filled circles represent the amino acids that are altered by the mutations found in the variant cell lines.

reduction in paclitaxel resistance (D.G., unpublished observations). Loss of paclitaxel resistance in this case, however, may be part of a pleiotropic phenomenon, because resistance to all six of the drugs tested were decreased in cells expressing the S222T mutation.

Analogous to the work carried out with steroid inhibitors, we have demonstrated that one can target the identification of mutations that reduce the efficacy of a taxane-based inhibitor, as well as resistance to the toxic form, paclitaxel. Thus, the data support the proposition that tRA-96023 interacts with the Pgp through determinants that are in common with paclitaxel. Of the six mutations identified through selections involving colchicine and tRA-96023, five exhibited phenotypic loss of tRA-96023 efficacy and paclitaxel resistance. Four of the mutations are located within the second half of the protein, and three are clustered within the TMS 10. One of the three (L865F) has been identified from the results of two independent selections. Another mutation (L998D) in TMS 12 was identified, through an additional selection, as a second mutation that acts in concert with L868W to cause a complete loss of paclitaxel resistance and more than a five-fold shift (relative to that in MS23 cells) in the efficacy of tRA-96023. The remaining mutation that affects the potency of both the inhibitor and paclitaxel (N350I) is located within the first half of the protein proximal to TMS 6. It results in the complete loss of paclitaxel resistance and

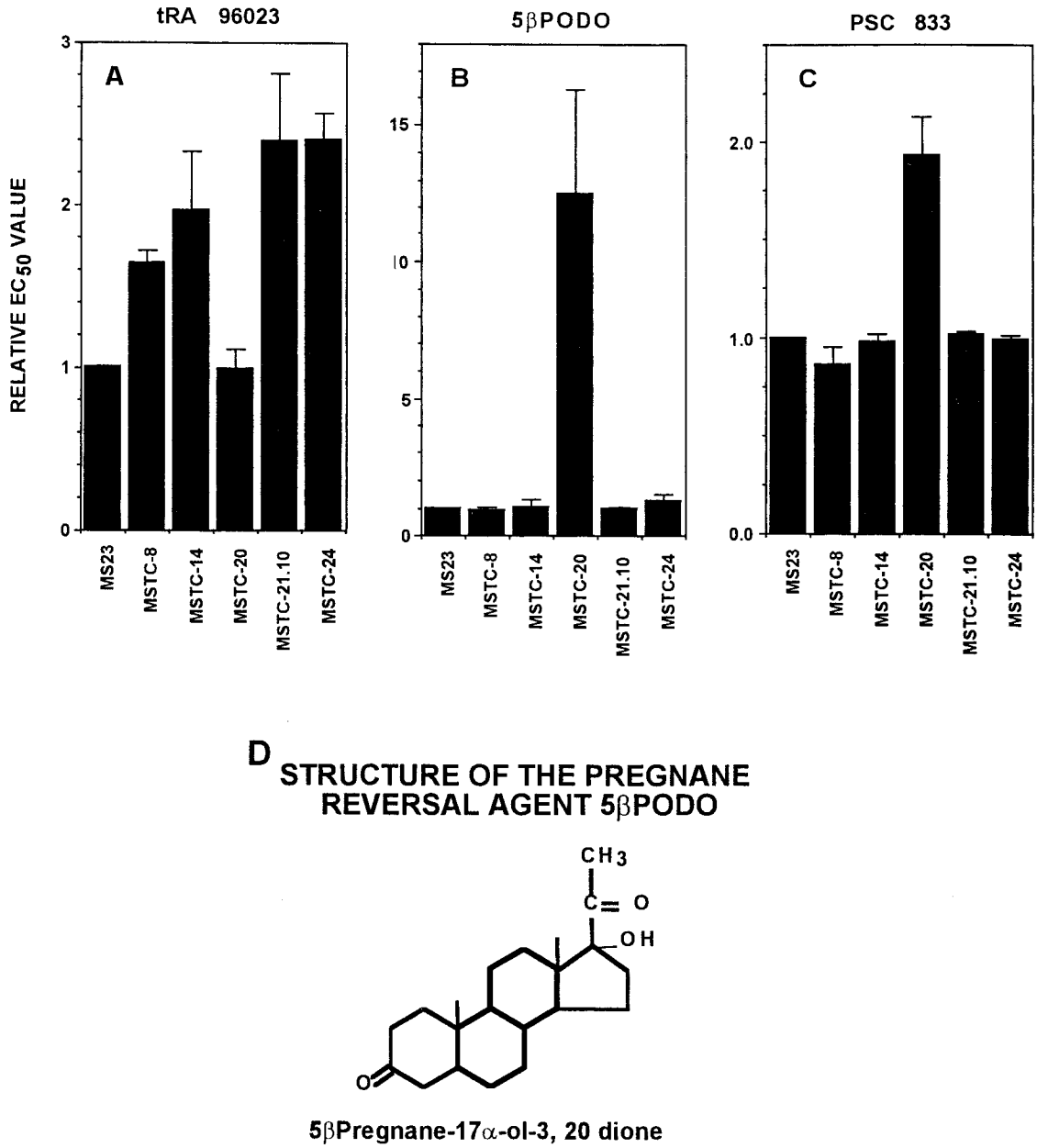

Fig. 4. Evaluation of P-glycoprotein inhibitor capacities in the MS23 and MSTC-8, -14, -20, -21.10, and -24 cell lines. Cell cultures, initially containing 5×10^4 cells/ml, were incubated in medium containing 5 μM puromycin and increasing concentrations of a P-glycoprotein inhibitor. At the end of the incubation period, the turbidities (660 nm) of the cultures were measured and expressed relative to cultures containing no drug. The concentration of inhibitor needed to reduce the turbidity value to 50% (EC_{50}) was estimated by interpolation. The EC_{50} values for the variant cell lines were normalized to that for the MS23 cell line. Each value represents the average and standard error for three independent experiments. A, tRA-96023; B, 5β-pregnane-17α-ol-3,20-dione; C, PSC 833.

a comparatively strong shift in the efficacy of tRA-96023 (Fig. 4). Interestingly, Wu et al. (1998) also observed photoaffinity labeling of a 95-kDa fragment that contained the six N-terminal TMS by the 3BzDC-modified analog of paclitaxel. They did not attribute the binding to a smaller subfragment, but the results do suggest that both halves of the protein can contribute to interactions with the taxanes. It is also worth noting that Loo and Clarke have reported evidence that TMS 6 interacts with TMS 10 to 12 (Loo and Clarke, 2000).

Pgp mutations may also produce effects that are not directly related to the binding and transport of drugs; the improper localization and insertion of the protein into the plasma membrane might be examples. Altered localization would reflect a change in the distribution of the Pgp between the plasma membrane and intracellular sites. Although the data presented here do not rule out such a possibility, the results in Table 2 show that there were no significant decreases in the resistance to daunomycin, dexamethasone (except for MSTC-20), and puromycin in the seven variant cell lines. Thus, if a mutation caused an increase in the relative number of intracellular sites, for instance, then Pgp localization at these sites would also need to have a capacity to convey resistance to the drugs listed above. Presumably, this could involve a sequestration of drugs that would reduce the concentrations available to interact with their intracellular targets. To our knowledge, this has not been demonstrated for puromycin and dexamethasone.

In contrast to the results described above, the Pgp mutation L338F, expressed in the MSTC-20 cells, had no effect upon the ability of tRA-96023 to inhibit drug transport (Fig. 4). L338F does, however, cause a profound change in steroid resistance and the inhibitory capacity of the steroid 5βPodo. This behavior and the location of the mutation within TMS 6 are consistent with the proposed steroid interactions with the TMS 4 to 6 region of the protein (Vo and Gruol, 1999). Moreover, the L338F mutation has been found expressed in a cell line independently selected with the combination of colchicine and a steroid inhibitor (D.G., unpublished observations). The resulting phenotype was identical to that seen with the MSTC-20 cells. Of the four of five that can be evaluated, none of the previously identified steroid-related mutations caused a significant loss of tRA-96023 activity (data not shown). This strongly suggests that steroids and taxanes interact with sites that are distinct from one another.

Although steroids and taxanes may interact with distinct sites, it is highly unlikely that the two domains function independently of one another. The obvious indication of this is that 5βPodo inhibits paclitaxel resistance and tRA-96023 inhibits dexamethasone resistance. Another line of evidence for a functional link between the two sites is provided by the 24TCTP-6 cell line. These cells express a Pgp with mutations in both TMS 10 (L868W) and TMS 12 (N988D). Acquisition of the second mutation (N988D) caused a three-fold increase in

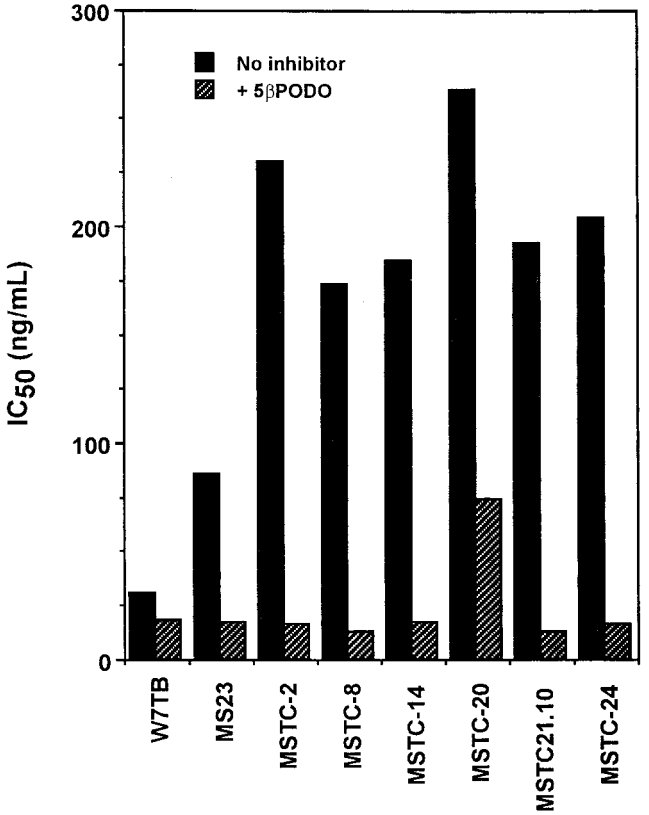

Fig. 5. Reversal of colchicine resistance by 5βPodo. Cell cultures, initially containing 5×10^4 cells/ml, were incubated in medium containing increasing concentrations of colchicine for 7 days. At the end of the incubation period, the turbidities (660 nm) of the cultures were measured and expressed relative to cultures containing no drug. The concentration of colchicine needed to reduce the turbidity value to 50% (IC_{50}) was estimated by interpolation. A parallel set of cultures were incubated with colchicine and 10 μM 5βPodo, and the IC_{50} values were determined.

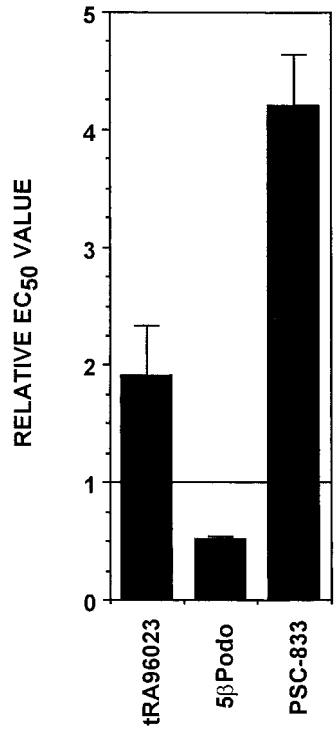

Fig. 6. Change in P-glycoprotein inhibitor efficacies due to the $N_{988}D$ mutation found in the 24TCTP-6 cells. MSTC-24 and 24TCTP-6 cells (5×10^4 cells/ml) were incubated for 7 days in the presence of 60 nM daunorubicin and increasing concentrations of the indicated P-glycoprotein inhibitor. At the end of the incubation period, the turbidities (660 nm) of the cultures were measured and expressed relative to cultures containing no inhibitor. The EC_{50} values obtained from the 24TCTP-6 cells were calculated and expressed relative to those of the MSTC-24 cells. The values represent the average and standard error for three independent experiments.

dexamethasone resistance along with an increased capacity of 5βPodo to reverse drug resistance. The latter phenomenon implies the enhanced ability of 5βPodo to interact with the Pgp and could reflect an increase in steroid binding affinity or an increase in site availability. Variations in either parameter would be an expected consequence of dynamic changes that normally take place in the TMS regions of the protein. Coupling between the two domains could be promoted by the TMS interactions (TMS 6 with TMS 10 to 12 and TMS 12 with TMS 4 to 6) that have been described by Loo and Clarke (2000). In the case described here, the mutation in the TMS 12 may be causing an alteration in the suppression of the steroid site. It should be noted that the increased resistance to dexamethasone and puromycin exhibited by the 24TCT-6 cell line is completely reversible by 10 μM verapamil, making it very unlikely that the phenotypic changes in this cell line are caused by changes other than the (N988D) mutation.

Our collection of variant cell lines derived from MS23 and expressing mutated Pgp also has the potential to contribute to the mapping of interaction domains of Pgp inhibitors other than those used in the original selections. This was illustrated in Figs. 4C and 6, which demonstrated that mutations in TMS 6 (L338F) and 12 (N988D) caused a reduction in the efficacy of the cyclosporin analog PSC 833. TMS 6 had been implicated in binding PSC 833 by Chen et al. (1997), who found that a TMS 6 F335 deletion in the human MDR1 Pgp caused reduced inhibition by PSC 833. Subsequently, Chen et al. (2000) showed that this mutation caused reduced photoaffinity labeling by cyclosporin and azidopine. In addition, Ma et al. (1997) presented evidence that point mutations in TMS 6 of the hamster Pgp1 gene caused a reduction in cyclosporin inhibitory activity. In this instance, two mutations contributed to the phenotype, G338A and A339P. Furthermore, Demuele et al. (1998) have used photoaffinity labeling to show that TMS 12 also participates in the interaction of cyclosporin with Pgp. Thus, the results of our studies are consistent with work of all three groups and the proposal that TMS 6 and 12 both contribute to Pgp interactions with PSC 833 and cyclosporin. Moreover, the results shown here indicate that the PSC 833 inhibitor interacts with regions of the Pgp that influence the interactions of both taxanes and steroids.

Acknowledgments

We thank Miranda King, Pak Leung, and Paula Pera for their expert technical assistance.

References

Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I and Gottesman MM (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol* **39:**361–398.

Ashida H, Oonishi T and Uyesaka N (1998) Kinetic analysis of the mechanism of action of the multidrug transporter. *J Theor Biol* **195:**219–232.

Bakos E, Klein I, Welker E, Szabo K, Muller M, Sarkadi B and Varadi A (1997) Characterization of the human multidrug resistance protein containing mutations in the ATP-binding cassette signature region. *Biochem J* **323:**777–783.

Bourgeois S, Gruol DJ, Newby RF and Rajah FM (1993) Expression of an *mdr* gene is associated with a new form of resistance to dexamethasone-induced apoptosis. *Mol Endocrinol* **7:**840–851.

Bradshaw DM and Arceci RJ (1998) Clinical relevance of transmembrane drug efflux as a mechanism of multidrug resistance. *J Clin Oncol* **16:**3674–3690.

Bruggemann EP, Germann UA, Gottesman MM and Pastan I (1989) Two different regions of phosphoglycoprotein are photoaffinity labeled by azidopine. *J Biol Chem* **264:**15483–15488.

Bruggemann EP, Currier SJ, Gottesman MM and Pastan I (1992) Characterization of the azidopine and vinblastine binding site of P-glycoprotein. *J Biol Chem* **267:**21020–21026.

Chen G, Duran GE, Steger KA, Lacayo NJ, Jaffrezou JP, Dumontet C and Sikic BI (1997) Multidrug-resistant human sarcoma cells with a mutant P-glycoprotein, altered phenotype, and resistance to cyclosporins. *J Biol Chem* **272:**5974–5982.

Chen GK, Lacayo NJ, Duran GE, Cohen D and Sikic BI (2000) Loss of cyclosporin and azidopine binding are associated with altered ATPase activity by a mutant P-glycoprotein with deleted phe(335). *Mol Pharmacol* **57:**769–777.

Choi K, Chen C-J, Kriegler M and Roninson IB (1988) An altered pattern of cross-resistance in multidrug-resistant human cells results from spontaneous mutations in the mdr1 (P-glycoprotein) gene. *Cell* **53:**519–529.

Currier SJ, Ueda K, Willingham MC, Pastan I and Gottesman MM (1989) Deletion and insertion mutants of the multidrug transporter. *J Biol Chem* **264:**14376–14381.

Demeule M, Laplante A, Murphy GF, Wenger RM and Beliveau R (1998) Identification of the cyclosporin-binding site in P-glycoprotein. *Biochemistry* **37:**18110–18118.

Demmer A, Thole H, Kubesch P, Brandt T, Raida M, Fislage R and Tummler B (1997) Localization of the iodomycin binding site in hamster P-glycoprotein. *J Biol Chem* **272:**20913–20919.

Dhir R, Grizzuti K, Kajiji S and Gros P (1993) Modulatory effects on substrate specificity of independent mutations at the serine939/941 position in predicted transmembrane domain 11 of P-glycoproteins. *Biochemistry* **32:**9492–9499.

Eytan GD and Kuchel PW (1999) Mechanism of action of P-glycoprotein in relation to passive membrane permeation. *Int Rev Cytol* **190:**175–250.

Greenberger LM (1993) Major photoaffinity drug labeling sites for iodoaryl azidoprazosin in P-glycoprotein are within, or immediately C-terminal to, transmembrane domains 6 and 12. *J Biol Chem* **268:**11417–11425.

Greenberger LM, Yang C-PH, Gindin E and Horwitz SB (1990) Photoaffinity probes for the α_1-adrenergic receptor and the calcium channel bind to a common domain in P-glycoprotein. *J Biol Chem* **265:**4394–4401.

Hafkemeyer P, Dey S, Ambudkar SV, Hrycyna CA, Pastan I and Gottesman MM (1998) Contribution to substrate specificity and transport of nonconserved residues in transmembrane domain 12 of human P-glycoprotein. *Biochemistry* **37:**16400–16409.

Hanna M, Brault M, Kwan T, Kast C and Gros P (1996) Mutagenesis of transmembrane domain 11 of P-glycoprotein by alanine scanning. *Biochemistry* **35:**3625–3635.

Harris AW, Bankhurst AD, Mason S and Warner NL (1973) Differentiated functions expressed by cultured mouse lymphoma cells. II. Theta antigen, surface immunoglobulin and a receptor on cells of a thymoma cell line. *J Immunol* **110:**431–438.

Higgins CF and Gottesman MM (1992) Is the multidrug transporter a flippase? *Trends Biochem Sci* **17:**18–21.

Kajiji S, Talbot F, Grizzuti K, Van Dyke-Phillips V, Agresti M, Safa AR and Gros P (1993) Functional analysis of P-glycoprotein mutants identifies predicted transmembrane domain 11 as a putative drug binding site. *Biochemistry* **32:**4185–4194.

Kane SE (1996) Multidrug resistance of cancer cells. *Adv Drug Res* **28:**181–252.

Kwan T and Gros P (1998) Mutational analysis of the P-glycoprotein first intracellular loop and flanking transmembrane domains. *Biochemistry* **37:**3337–3350.

Loo TW and Clarke DM (1993) Functional consequences of phenylalanine mutations in the predicted transmembrane domain of P-glycoprotein. *J Biol Chem* **268:**19965–19972.

Loo TW and Clarke DM (1996) Mutational analysis of the predicted first transmembrane segment of each homologous half of human P-glycoprotein suggests that they are symmetrically arranged in the membrane. *J Biol Chem* **271:**15414–15419.

Loo TW and Clarke DM (1997) Identification of residues in the drug-binding site of human P-glycoprotein using a thiol-reactive substrate. *J Biol Chem* **272:**31945–31948.

Loo TW and Clarke DM (1999) Determining the structure and mechanism of the human multidrug resistance P-glycoprotein using cysteine-scanning mutagenesis and thiol- modification techniques. *Biochim Biophys Acta* **1461:**315–325.

Loo TW and Clarke DM (2000) The packing of the transmembrane segments of human multidrug resistance P-glycoprotein is revealed by disulfide cross-linking analysis. *J Biol Chem* **275:**5253–5256.

Ma JF, Grant G and Melera PW (1997) Mutations in the sixth transmembrane domain of P-glycoprotein that alter the pattern of cross-resistance also alter sensitivity to cyclosporin A reversal. *Mol Pharmacol* **51:**922–930.

Morris DI, Greenberger LM, Bruggemann EP, Cardarelli C, Gottesman MM, Pastan I and Seamon KB (1995) Localization of the forskolin labeling sites to both halves of P-glycoprotein: similarity of the sites labeled by forskolin and prazosin. *Mol Pharmacol* **46:**329–337.

Ojima I, Bounaud PY, Takeuchi C, Pera P and Bernacki RJ (1998) New taxanes as highly efficient reversal agents for multidrug resistance in cancer cells. *Bioorg Med Chem Lett* **8:**189–194.

Raviv Y, Pollard HB, Bruggemann EP, Pastan I and Gottesman MM (1990) Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells. *J Biol Chem* **265:**3975–3980.

Rosenberg MF, Callaghan R, Ford RC and Higgins CF (1997) Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. *J Biol Chem* **272:**10685–10694.

Shapiro AB, Corder AB and Ling V (1997) P-glycoprotein-mediated Hoechst 33342 transport out of the lipid bilayer. *Eur J Biochem* **250:**115–121.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* **82:**1107–1112.

Taguchi Y, Kino K, Morishima M, Komano T, Kane SE and Ueda K (1997) Alteration of substrate specificity by mutations at the His61 position in predicted transmembrane domain 1 of human MDR1/P-glycoprotein. *Biochemistry* **36:**8883–8889.

Vo QD and Gruol DJ (1999) Identification of P-glycoprotein mutations causing a loss of steroid recognition and transport. *J Biol Chem* **274:**20318–20327.

Wu Q, Bounaud PY, Kuduk SD, Yang CP, Ojima I, Horwitz SB and Orr GA (1998) Identification of the domains of photoincorporation of the 3′-and 7-benzophenone analogues of Taxol in the carboxyl-terminal half of murine mdr1b P-glycoprotein. *Biochemistry* **37:**11272–11279.

Zhang X, Collins KI and Greenberger LM (1995) Functional evidence that transmembrane 12 and the loop between transmembrane 11 and 12 form part of the drug-binding domain in P-glycoprotein encoded by. *MDR*1. *J Biol Chem* **270:**5441–5448.

Address correspondence to: Dr. Donald Gruol, The Sidney Kimmel Cancer Center, 10835 Altman Row, San Diego, CA 92121. E-mail: dgruol@skcc.org