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Evidence for the Locations of Distinct Steroid and Vinca Alkaloid Interaction Domains within the Murine mdr1b P-Glycoprotein

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ABSTRACT

P-glycoproteins (P-gp) cause the efflux of a wide variety of unrelated hydrophobic compounds out of cells. However, the locations of the sites at which different classes of molecules initially interact with the protein are not well defined. A unique system was developed to search for P-gp drug-interaction domains using mutational analysis. The strategy is based upon identifying mutations that cause a decrease in the activity of P-gp inhibitors, which are structurally related to chemotherapeutic drugs transported by P-gps. Evidence of distinct steroid and taxane interaction domains has already been presented. The work reported here extends the study of the steroid interaction domain and presents evidence for a separate vinblastine interaction domain. A total of 10 steroid-related mutations, involving seven amino acids that are confined within transmembrane segments (TMS) 4 to 6, have been characterized. The location of these mutations indicates that steroids interact with the transporter within the inner leaflet of the plasma membrane. Four previously unidentified, Vinca-related mutations, involving three amino acids, have also been found. Unexpectedly, these mutations are clustered within an eight-amino acid segment proximal to the TMS-4 region. This portion of the protein is thought to be within the cytoplasmic compartment of the cell. Thus, the results suggest that at least part of the initial interaction between P-gp and Vinca alkaloids occurs in the cytoplasm. The steroid interaction domain does not extend into this region of the protein. However, this cytoplasmic section of the protein is likely to play an important role in promoting steroid transport.

Expression of ATP binding cassette drug transporters can convey cellular multidrug resistance to organisms as diverse as bacteria and man (van Veen and Konings, 1997). P-glycoproteins are members of the ATP binding cassette family whose expression can compromise the efficacy of chemotherapy (Gottesman et al., 2002). This is accomplished through their ability to cause an efflux of many different amphipathic molecules (Ambudkar et al., 1999). To fully understand how this is accomplished, it is necessary to know where drugs make their initial interactions with the protein. A current model of P-gp function proposes that drugs are intercepted as they traverse the plasma membrane (Shapiro and Ling, 1998; Eytan and Kuchel, 1999; Chen et al., 2001). This leads to their translocation from the inner lipid leaflet to the external aqueous environment (Raviv et al., 1990) or their being "flipped" back into the outer lipid leaflet of the bilayer (Higgins and Gottesman, 1992). There is considerable evidence that half of the transmembrane segments of the protein (TMS 4–6 and TMS 10–12) participate in drug binding (Bruggemann et al., 1989; Greenberger et al., 1990; Greenberger, 1993; Zhang et al., 1995). The key question is whether there is a single drug binding pocket with limited specificity or an extended drug-binding region with distinct sites that exert an allosteric influence on each other's activity?

P-glycoproteins are organized into two structurally related halves, each containing six TMS and a cytoplasmic ATP-binding domain. This arrangement may be the result of tandem duplication (Raymond and Gros, 1989) or the fusion of two related transporters (Chen et al., 1990). A model of the protein's proposed organization relative to the plasma membrane is provided under *Results*. Rosenberg et al. (2001) used two-dimensional crystals of purified P-gp (Chinese hamster ovary cells) and electron cryomicroscopy to obtain detailed structural information about the transporter. The resulting model indicates that the TMS participate in the formation of a bipartite structure. Without ATP, this structure resembles a truncated inverted cone that is closed at the cytoplasmic interface. The interior of the cone forms a chamber that is accessible to the external environment. Such a configuration

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ABBREVIATIONS: P-gp, P-glycoprotein; TMS, transmembrane segment; BODIPY, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid; BV, BODIPY-vinblastine; 5βPodo, 5β-pregnane 17α-ol-3,20 dione.

is consistent with portions of the TMS that are nearest to the cytoplasm being near one another. This type of arrangement was predicted for the TMS 4 to 6 and 10 to 12 regions as a result of P-gp cross-linking studies (Loo and Clarke, 1999).

Photoaffinity labeling has been used to explore the location of binding sites for a variety of compounds. Although the results demonstrate the potential for distinct sites, they also highlight the complexity that is likely to be involved. There is evidence for an anthracycline site within the first half of the protein (Demmer et al., 1997) and a taxane site within the second half of the protein (Wu et al., 1998). These results are consistent with the bipartite P-gp structure seen by Rosenberg et al. (2001). Iodoprazosin may have separate binding sites within both halves of the protein (Dey et al., 1997; Isenberg et al., 2001) and the azidopine binding site may involve elements from both halves of the protein (Bruggemann et al., 1992; Morris et al., 1995). Several other approaches have yielded evidence of distinct drug binding sites but were not designed to indicate the location of the sites within the protein (Tamai and Safa, 1991; Spoelstra et al., 1994; Ayesh et al., 1996; Pascaud et al., 1998; Shapiro et al., 1999). These studies were based on evaluating the effect of one drug on a second drug's interaction with P-gp. For instance, Martin et al. (2000) used equilibrium and kinetic radioligand binding to purified plasma membrane samples containing P-gp. Based upon evidence of noncooperative interactions between the binding of a series of different drugs, four separate binding sites were proposed for vinblastine, paclitaxel, Hoechst 33342, and nicardipine. Ferry et al. (1995) had also used binding kinetics to obtain evidence of separate sites for vinblastine and nicardipine.

Changes in the drug resistance profiles of cells expressing mutated P-gp have often been referred to as a change in specificity. However, changes in specificity do not necessarily reflect alterations on drug binding (Beaudet et al., 1998). Drug resistance reflects the culmination of a series of steps including drug binding, ATP binding, ATPase activation, and drug translocation and release. A mutation altering any one of these steps could change the drug resistance profile of the cell. Inhibition of drug transport, on the other hand, is thought to reflect the consequence of an inhibitor being involved in only the first step, binding to the protein. Thus, mutations that cause a decrease in the activity of a specific inhibitor are more likely to reflect a change in the binding of the inhibitor to the protein. The work presented here demonstrates how this concept can be employed to achieve a targeted identification of mutations reflecting the location of distinct interaction domains for steroids and Vinca alkaloids. Moreover, variant cells expressing altered P-gp can be used to study the effects of mutations in one domain on the function of other domains.

Materials and Methods

Cell Culture. WEHI-7 is a thymoma cell line obtained from a female BALB/c mouse after exposure to X-irradiation (Harris et al., 1973). Corticosteroids induce apoptosis in WEHI-7 cells. 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 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 as described in Vo and Gruol (1999). 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 pair of selective drugs. The dual-drug selections contain a toxic drug and either a steroid or vinblastine-based P-glycoprotein inhibitor. The MEK-417 compound is a congener of the series of vinblastine-based diastereomeric inhibitors described by Borman et al. (1993), and its structure is provided under *Results*. The dual-drug selections were carried out under the following conditions: 20 μM 5β-androstan-17α-ol-3-one and 50 ng/ml of colchicine; 10 μM 5β-pregnane-17α-ol-3, 20 dione and 40 nM of paclitaxel; 15 μM of 5β-pregnane-17α-ol-3,20 dione and 45 nM of daunomycin; 5β-androstan-17α-ol-3-one, 1.5 μM RU-486, and 5 μM of puromycin; 1.8 μM MEK-417 and 54 ng/ml of colchicine. Resistant colonies typically appeared between 12 to 18 days. Each of the variant lines was initially screened for changes in growth in the presence of either dexamethasone or vincristine relative to MS23 cells. The results of these evaluations identified those variants that had lost significant steroid or Vinca alkaloid resistance.

Evaluation of Drug Resistance. The effect of drugs on 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 normalizing the values 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 where 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 fractional change in resistance for a toxic drug and a given variant cell line is determined by measuring the IC_{50} for the variant cell line and using the IC_{50} values obtained from the W7TB and MS23 cell lines as standards. The IC_{50} for the MS23 cells is subtracted from the IC_{50} of the variant cells. This value is divided by the difference in IC_{50} values between the MS23 and W7TB cell lines. The equation is presented with Table 1.

Evaluation of Inhibitor Efficacy. 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 efficiency of the inhibitor is reflected by an EC_{50} value, which is defined as the concentration of P-gp inhibitor that reduces the relative turbidity value to 50%.

Evaluation of *mdr*1 P-glycoprotein Mutations Expressed in the MS23 Variants. Reverse transcription-polymerase chain reaction 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 generated using a set of nested primers. The individual polymerase chain reaction 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).

TABLE 1

Summary of the changes in the drug resistance profiles for variants expressing the steroid-related mutations

The effects of the listed drugs on cell growth was determined for the W7TB, MS23, and variant cell lines as described under *Materials and Methods*. These measurements allowed the evaluation of an IC_{50} value that reflects the reduction in cell growth by 50%. The change in IC_{50} values between a variant line and MS23 is normalized to the difference in IC_{50} values between W7TB and MS23. The percentage of normalized change in resistance (N.C.R) is defined by the equation: $N.C.R = 100\% \times ({}^{VAR}IC_{50} - {}^{MS23}IC_{50})/({}^{MS23}IC_{50} - {}^{W7TB}IC_{50})$

Cell Line	Vincristine	Daunomycin	Dexamethasone	Puromycin	Colchicine	Mutation
MSPD-10	N.C.	▼	▼▼▼	▼▼	▼▼	W231C
MSPT-7	▼▼	▼▼▼	▼▼▼	▼▼▼	▼	A229V, W231L
MSPD-60	▼▼	N.C.	▼▼▼	▼▼	▼▼	A301D
MSMP-1	▼	▼▼	▼▼	▼▼	▼▼▼	S308L
MSAC-52	▼	N.C.	▼▼	N.C.	++++	L338F

N.C., no change; ++++, NCR increase ≥200%; ▼, NCR decrease ≥33%; ▼▼, ≥67%; ▼▼▼, ≥100%.

Evaluation of Intracellular Drug Accumulation. The ability of cells to accumulate either daunomycin or BODIPY-vinblastine (BV) was measured by flow cytometry using a Becton Dickinson FACScan instrument. Daunomycin fluorescence was measured by the FL-2 detector and BV fluorescence was measured by the FL-1 detector. These data were employed to generate histograms reflecting the range of drug content per cell. The geometric mean of the population was used to characterize the drug content.

Chemicals. The steroids 5β-pregnane-17α-ol-3,20-dione (5βPodo) and 5β-androstan-17α-ol-3-one were obtained from Steraloids Inc. (Newport, RI). Dexamethasone, paclitaxel, vincristine, puromycin, daunomycin, verapamil, and colchicine were obtained from Sigma (St. Louis, MO). BODIPY-vinblastine was obtained from Molecular Probes Inc. (Eugene, OR).

Results

The combination of a toxic drug and an unrelated P-gp inhibitor can be used to select for variant cell lines expressing mutated P-gp (Vo and Gruol, 1999; Gruol et al., 2001). This strategy is directed at identifying mutations that alter the ability of a specific class of inhibitor to interact with the protein. Moreover, when the inhibitor is a nontoxic homolog of a transported toxic drug, the selected mutations should also reduce resistance to the toxic form. We previously reported the use of a pregnane steroid inhibitor, 5βPodo, in combination with puromycin to isolate variant cell lines that each expressed one of five different mutations in the mdr1b P-gp (Vo and Gruol, 1999). These murine thymoma cell lines exhibited a decrease in 5βPodo inhibitor activity, as well as a complete reversal of dexamethasone resistance. Each one of the variant cell lines, however, also exhibited a partial loss in puromycin resistance. This behavior suggested that additional mutations might exist that alter the interaction of P-gp with steroids, which cause a more complete reversal of puromycin resistance. Variants expressing such P-gp mutations would not survive selections employing puromycin as the toxic drug. Therefore, we wished to test whether the five previously identified steroid-related mutations were representative of those that can be found by dual-drug selections. Additional selections, involving different combinations of toxic drugs (daunomycin, colchicine, and paclitaxel) and steroids (5β-androstan-17α-ol-3 one, RU486, and 5βPodo) were carried out. The details of the selections are described under *Materials and Methods*. Figure 1 depicts the structures of the three steroids, which were used in the selections, compared with the toxic form dexamethasone.

Table 1 depicts the changes in the multidrug resistance profiles of five new variants expressing mutated mdr1b P-gp that were obtained from five independent selections. All of these mutations cause a >90% loss of dexamethasone resistance. Four of the six mutations caused a >67% loss of puromycin resistance. The MSPD-10 and MSPD-60 cell lines were isolated from selections involving 5βPodo and daunomycin. Each expresses a new mutation that involves an amino acid that was previously found to be mutated to a different form (Vo and Gruol, 1999). The W231C mutation occurs in the same residue as a W231L mutation. The A301D mutation occurs in the same residue as the A301T and A301V mutations. The MSPT-7 variant was isolated from a selection involving 5βPodo and paclitaxel. It contains two closely spaced mutations, A229V and W231L. As indicated above, the W231L mutation had previously been identified. The MSAC-52 variant was isolated from selections involving 5β-androstan-17α-ol-3 one and colchicine. The L338F mutation (MSAC-52) had been previously identified through a selection involving a taxane-based inhibitor and colchicine (Gruol et al., 2001). Both variant cell lines expressing the L338F mutation exhibit very similar profiles of drug resistance. The MSMP-1 variant was isolated from a selection involving puromycin and two steroid P-gp inhibitors, 5β-androstan-17-α-ol-3 one and RU486. This line expresses the S308L mutation that had not been identified previously. Another variant expressing the S308L mutation was independently isolated employing a selection involving 5β-androstan-17-α-ol-3 one and paclitaxel. In total, the process identified five new mutations, two of which (A229V, S308L) involve amino acids that had not been previously identified as contributing to steroid interactions with the P-gp.

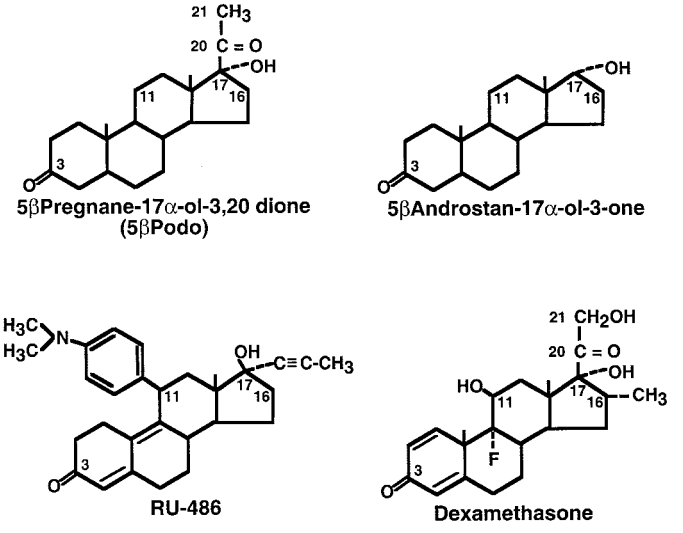

Fig. 1. The structures of three steroid P-glycoprotein inhibitors compared with that of dexamethasone.

Figure 2 depicts the seven different locations associated with all 10 steroid-related mutations, as they exist within the proposed relationship of the P-gp with the plasma membrane. The mutations are located within the transmembrane segments (TMS) 4 to 6, and all seven are associated with positions that are likely to be located within, or near, the inner leaflet of the membrane bilayer. The apparent "clustering" of these 10 mutations suggests that the TMS-4, -5, -6 region of the protein participates in forming a structure that provides for the specific binding of this class of compounds. If this interpretation is correct, all of the mutations should cause a reduction in the inhibitory activity of 5βPodo. One feature of the drug resistance profiles is that only 1 (MSPD-60) of five new variant cell lines exhibits a substantial (>33%) reduction in paclitaxel resistance. Moreover, none of the five previously reported steroid-related mutations (Vo and Gruol, 1999) caused a significantly altered paclitaxel resistance (defined as a loss of more than 33% or a gain of resistance of 50%). This behavior is illustrated for all 10 variant cell lines in Fig. 3A. The limited change in paclitaxel resistance provides the means to evaluate the relative ability of 5βPodo to reverse nearly equivalent levels of paclitaxel resistance in nine of the variants. Figure 3B illustrates the results of an experiment where the MS23 line (normal P-gp) and the nine mutation-expressing variants were grown in a nontoxic concentration (40 nM, for cells expressing P-gp) of paclitaxel. The inhibitor 5βPodo was titrated into a series of cultures to determine the effect of the different mutations upon the ability of the steroid to reverse drug resistance. An EC_{50} value was determined for each cell line. It represents the concentration of inhibitor needed to reverse the paclitaxel resistance by 50%. The relative EC_{50} values were obtained by normalizing the EC_{50} values of the variants to that of the MS23 cells. The degree to which the relative EC_{50} values are greater than 1.0 is representative of a reduction in the effectiveness of the steroid inhibitor 5βPodo. The values for the nine steroid-related mutations ranged from 9.3 to 144-fold. The largest increases were displayed by the mutations located within TMS-4. The relative EC_{50} values associated with the TMS-4 variants expressing single-point mutations (MSPP-21, MSPP-1, and MSPD-10) range from 27 to 60. The double mutation expressed in the MSPT-7 cells shifts the relative EC_{50} value to 144. The mutations in TMS-5 (MSPP-4, MSPP-17, and MSMP-1) and TMS-6 (MSAC-52, MSPP-6) cause values ranging from 9.3 to 16.3. Daunomycin was used to evaluate the effect of the A301D mutation, expressed in the MSPD-60 cells, on the inhibitory activity of 5βPodo. In this instance, the relative EC_{50} value was more than 60-fold. A more precise measurement was not possible because of the concentration of 5βPodo necessary to reverse daunomycin resistance in the MS23 cells (~0.5 μM) and the limiting concentration that can be employed (30 μM) without producing nonspecific effects upon cell growth.

The effect of the mutations upon the capacities of other classes of inhibitors to reverse drug resistance can also be evaluated. This test provides a valuable measure of the selectivity of the mutations for interfering with steroid-P-gp interactions. Figure 4A depicts the effects of the nine mutations on the ability of verapamil to reverse paclitaxel resistance. Seven of the mutations caused no significant effect. The changes associated with the double mutation expressed in MSTP-7 and the single mutation expressed in the MSMP-1 cells were modest (2.6 and 3.1, respectively) compared with those observed in Fig. 3B. Thus, the mutation-induced changes in inhibitor activity are far more pronounced for the steroid inhibitor. This calls into question whether verapamil

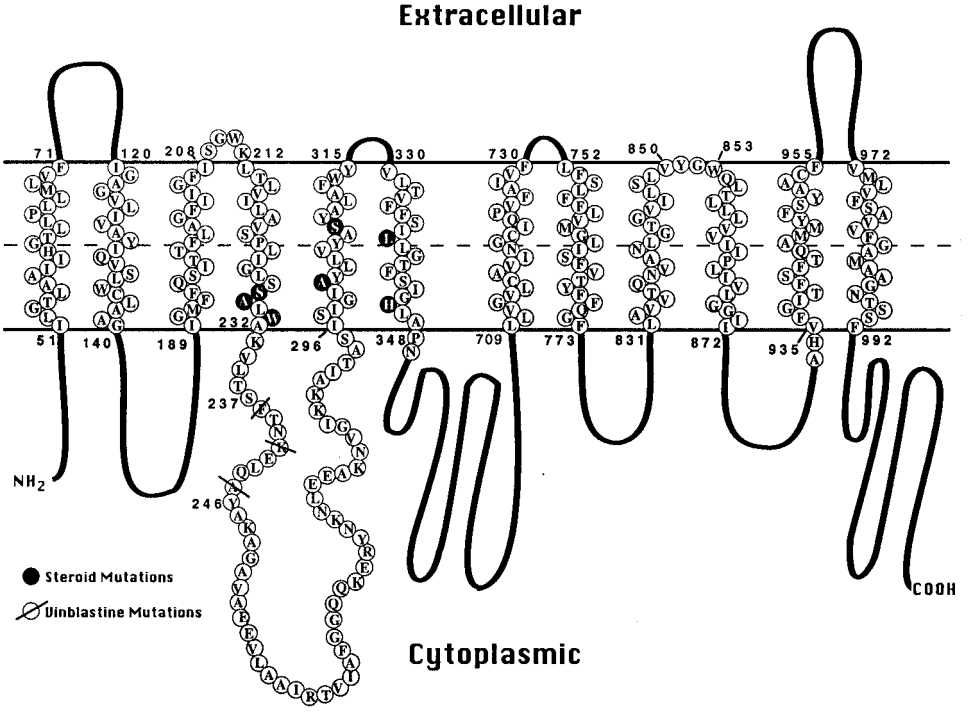

Fig. 2. The location of two sets of P-glycoprotein mutations within the proposed relationship between the protein and the plasma membrane. The filled circles represent the amino acids that are altered by the steroid-related mutations. The slashed circles represent the amino acids that are altered by the Vinca-related mutations. The amino acids proposed to be located within the plasma membrane encompass ~20% of the protein.

Steroid-related mutations: S228T; W231L; W231C; A229V, W231L; A301V; A301T; A301V; S308L; L338F; H346N
Vinca-related mutations: F238S; K241N; K241T; A245D

interacts within the TMS 4–6 portion of the protein. It should be noted that Loo and Clarke (2001) also tested one of the 10 steroid-related mutations, W231C (MSPD-10 cells), for its effect on the human MDR1 P-gp's interaction with verapamil. In the human protein, the comparable mutation is W232C. These studies were carried out by evaluating the ability of a modified form of the inhibitor, methanethiosulfonate-verapamil, to induce P-gp ATPase activity in an in vitro assay. The results obtained with the human and murine proteins are in agreement; there was no change in activity. However, the human L339C mutation produced a nearly complete loss of the ability of methanethiosulfonate-verapamil to induce ATPase activity. In contrast, a mutation at the comparable position in the murine protein, L338F (MSAC-52 cells), had no effect on the ability of the inhibitor to reverse paclitaxel resistance. It is not clear whether the disparity in the results reflects the contrasting forms of the mutation, the different forms of verapamil, or the two types of assay used to evaluate the changes in activity.

Not all steroids are P-gp inhibitors, and not all steroid-based inhibitors seem to interact within the same domain, as does 5βPodo. The activity of the steroid inhibitor RU486 (Gruol et al., 1994) was also evaluated for the variants expressing the 10 steroid-related mutations. Nine of the 10 variants exhibited no significant increase in the relative EC_{50} value (Fig. 4B). Only the double mutation (A229, W231L) expressed in the MSPT-7 cells caused modest decrease in inhibitory activity, yielding an EC_{50} value of 2.7. The most pronounced effect was caused by the S228T mutation expressed in the MSPP-21 cells. Instead of a loss of

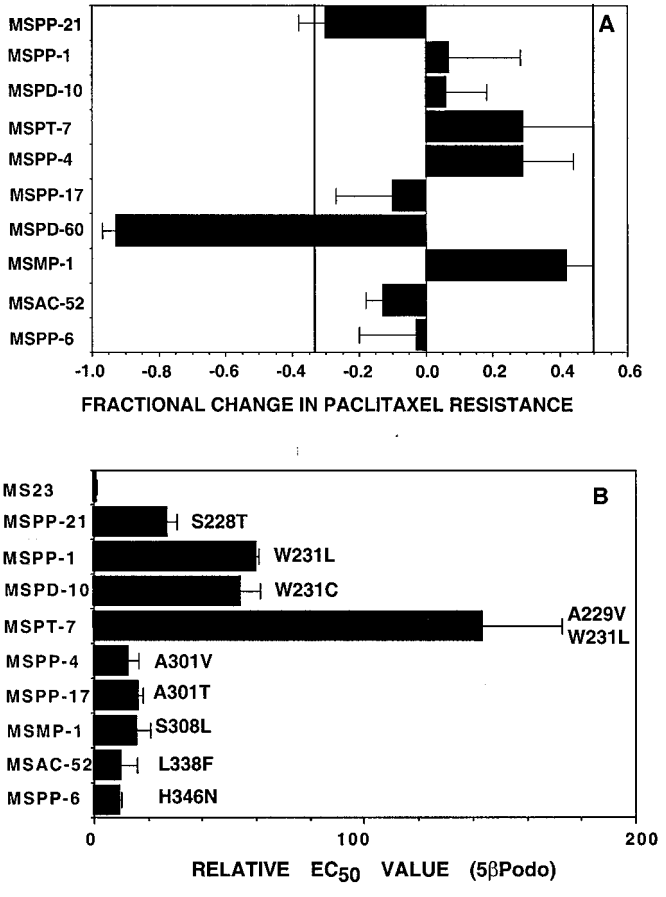

Fig. 3. The effects of the steroid-related mutations upon paclitaxel resistance and 5βPodo inhibitor activity. A, cell cultures of W7TB, MS23, and the steroid-related variants were incubated with increasing concentrations of paclitaxel to evaluate the IC_{50} values as outlined under *Materials and Methods*. The difference in the IC_{50} values obtained for W7TB and MS23 was designated as the degree of paclitaxel resistance (DPR) value. The shift in paclitaxel resistances for the variants were obtained by evaluating the difference between the IC_{50} value for a given variant and for MS23 (ΔR). The fractional change in paclitaxel resistance for a given variant is defined as ΔR/DPR. These values can be negative or positive depending upon whether the variant has lost or gained resistance to paclitaxel relative to the MS23 cells. These fractional values, converted to percentages, are the normalize change in resistance values described in Table 1. B, cell cultures of MS23 and nine of the variants expressing the steroid-related mutations were incubated with a fixed concentration of paclitaxel (40 nM) and increasing concentrations of 5βPodo for 7 days. The relative EC_{50} values were determined as described under *Materials and Methods*.

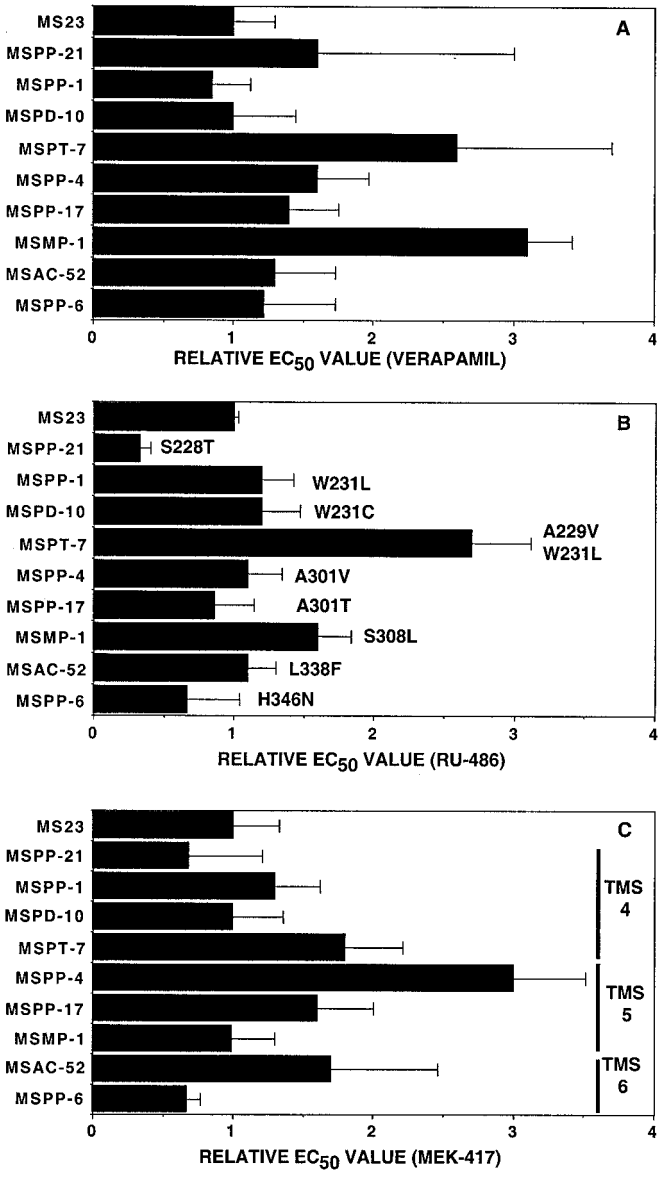

Fig. 4. The effects of the steroid-related mutations on the inhibitory activities of verapamil and the vinblastine-based inhibitor MEK-417. The relative EC_{50} values for verapamil (A), RU-486 (B), and MEK-417 (C) were determined for the variants expressing the steroid-related mutations as in Fig. 3B. Figure 4, B and C, also contain an indication of the locations of the mutations expressed in the variant cell lines. The locations are designated as the amino acid changes or TMS-4, -5, -6, which refer to transmembrane segments of the P-gp.

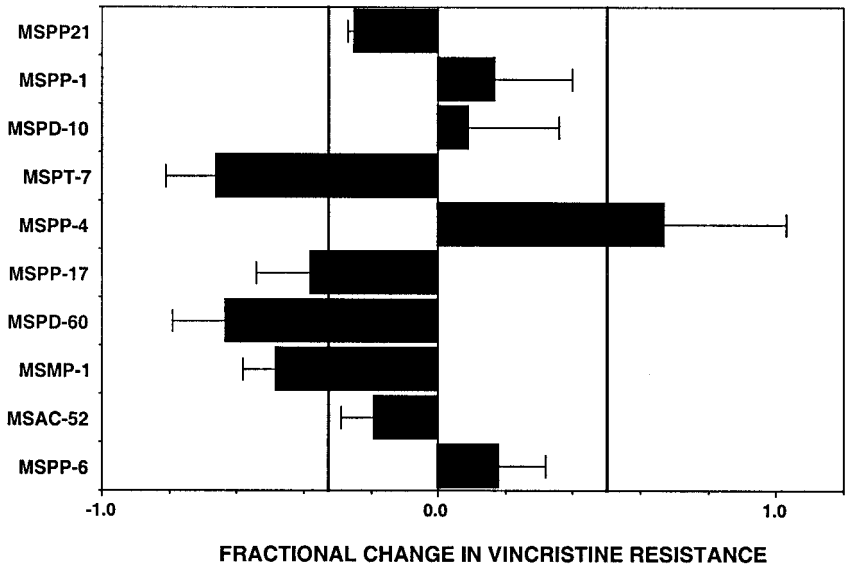

Fig. 5. The effects of the steroid-related mutations on the change in vincristine resistance. The fractional change in vincristine resistance was evaluated for the variants expressing the steroid-related mutations as described for Fig. 3A.

inhibitory activity, RU-486 was 3-fold more effective in these cells. Taken together, the results do not indicate that RU486 interacts at the same site as 5βPodo.

Four of the steroid-related mutations cause a substantial reduction in vincristine resistance. This is demonstrated in Fig. 5. This observation raises the issue whether Vinca alkaloids interact within the same region of the protein as steroids. Figure 4C depicts an evaluation of the effects of the steroid-related mutations on the activity of the P-gp inhibitor MEK-417. The MEK-417 compound is a congener of the vinblastine-based diastereomeric inhibitors described by Borman et al. (1993). The structure of this inhibitor will be presented below. Again, the effects of the steroid-related mutations are relatively modest. The mutation expressed in the MSPP-4 cells (A301V) shows the largest reduction of MEK-417 inhibitory activity. However, Fig. 5 demonstrates that the resistance to vincristine increases in the MSPP-4 cells. Thus, it seems unlikely that the (A301V) mutation interferes with the initial binding of Vinca alkaloids with the protein. It is possible that A301V promotes the transport of MEK-417 also, which would lead to lower inhibitory activity. Conversely, three of the variants (MSPT-7, MSPP-17, and MSMP-1) exhibit a significant reduction in vincristine resistance, which are without an accompanying substantial change in the vinblastine-based inhibitor's activity. We conclude that these combinations of phenotypic changes are inconsistent with any of the 10 mutations altering the initial interaction of Vinca drugs with the protein.

To establish where Vinca alkaloids might interact with P-gp, dual-drug selections similar to those made with steroid inhibitors were carried out with the combination of the inhibitor MEK-417 and colchicine. Figure 6 depicts the structures of vinblastine and its nontoxic congener MEK-417. Similar to the approach employed to identify the steroid-related mutations, MS23 samples were mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, allowed to recover and placed under selection by the combination of colchicine and the inhibitor. Surviving clones were obtained with frequencies of 7.5×10^{-7} and 2×10^{-7} from two independent selections. Approximately half of these clones exhibited a significant reduction in vincristine resistance and were analyzed further. Variants expressing four P-gp mutations were found, none of which had been identified previously. Table 2 lists the variant cell lines, the mutations, and the changes in their drug resistance profiles. The four mutations involve three amino acids, which are clustered within an eight-residue segment. This region of the protein has been proposed to be located within the cytoplasmic compartment, proximal to TMS 4 (see Fig. 2). All four of the mutations caused a reduction in the resistance to vincristine, paclitaxel, and dexamethasone. None cause a loss of resistance to puromycin, daunomycin, or colchicine. The large increase in colchicine resistance observed in the MSVbC-32 cells can be completely reversed by verapamil (data not shown). This indicates that the increased colchicine resistance is caused by enhanced P-gp capacity to transport colchicine in the MSVbC-32 cells. The other three variants exhibit a large increase in etoposide resistance (data not shown).

The selected loss of vincristine resistance in cell lines expressing mutated Pgp indicates altered drug transport leading to increased Vinca alkaloid accumulation. This behavior can be confirmed by evaluating the effect of the mutations on the intracellular accumulation of BODIPY-vinblastine, a fluorescent vinblastine derivative that has been demonstrated to be a substrate for P-gp (Kolchinsky and Roninson, 1997). The accumulation of BV can be measured using flow cytometry. The results of such a study are depicted in Fig. 7. Cultures of W7TB, MS23, and the four variant cell lines expressing the Vinca-related mutations were incubated with 15 nM BV and tested for their ability to retain the drug. Histograms of the data indicated single component distributions and the geometric means of the distributions were used to characterize the BV accumulation in each cell line. Expression of the normal P-gp, in the MS23 cell line, caused a 50% reduction of BV compared with that observed for the W7TB cells (no P-gp). In contrast, all four of the mutated forms of the protein allowed a significantly increased BV accumulation, relative to the MS23 cells. Moreover, none of the variant cell lines exhibited increased levels of daunomycin accumulation (data not shown), which indicates that the effects on BV accumulation are drug-specific.

Figure 8 illustrates the effects of the Vinca-related muta-

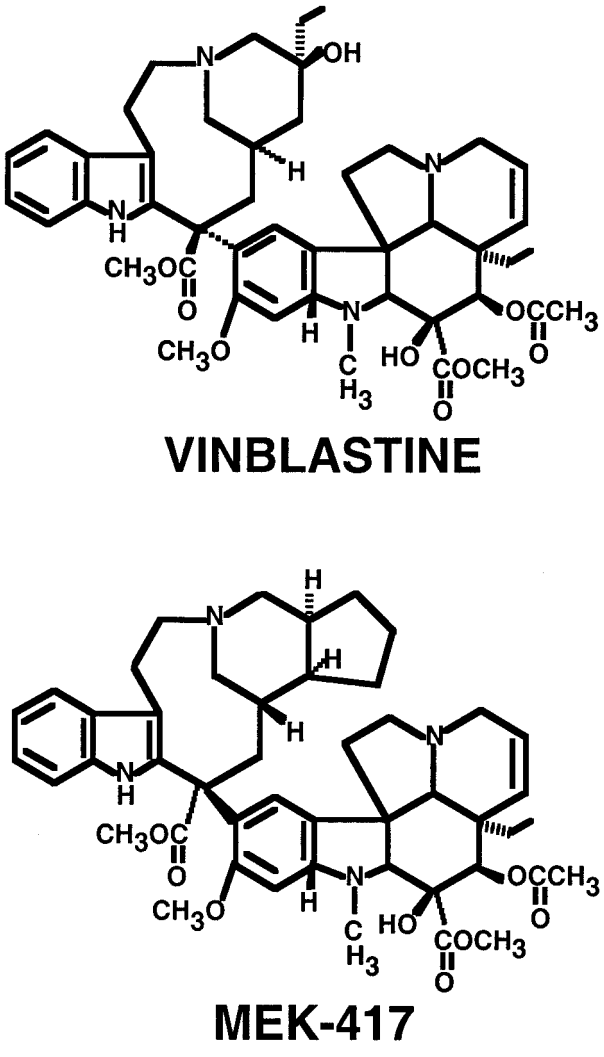

Fig. 6. The structures of vinblastine and MEK-417.

tions on the relative abilities of three P-gp inhibitors to reverse puromycin resistance. The variant cell lines expressing the mutated P-gp were incubated with a fixed concentration of puromycin and the EC_{50} values for the vinblastine-based inhibitor MEK-417, the steroid inhibitor 5βPodo, and verapamil were measured. The increase in relative EC_{50} values depicted in the left is indicative of a reduction in MEK-417 inhibitory activity. This behavior, and the loss of vincristine resistance in the variant cell lines (Table 2), is consistent with a reduced interaction between Vinca alkaloids and the protein. The magnitude of the changes in the relative EC_{50} values for MEK-417 are smaller than those observed for the steroid inhibitor 5βPodo and the 10 steroid-related mutations (Fig. 3). We believe that this can be attributed to the fact that the MEK-417 molecule is twice the molecular weight of 5βPodo and potentially capable of having a more complex set of interactions with the protein. A single point mutation may not disrupt the MEK-417/P-gp interaction as completely as is possible with the simpler steroids. Fig. 8, right, demonstrates that the four mutations had very little effect on the inhibitory activity of verapamil (please note the change in scale). The results presented in Fig. 8, middle, reflect the effect of the mutations on the inhibitory activity of 5βPodo. The data reveal that three of the mutations cause a significant increase in steroid inhibitor activity (decrease in relative EC_{50} value). The greatest effect is associated with the A245D mutation expressed in the MSVbC-32 cell line. There is a more than 90% decrease in the EC_{50} value, 590 nM for MS23 versus 54 nM for MSVbC-32, in this variant cell line. Conversely, the mutations all cause a reduction in dexamethasone resistance (Table 2), indicating that steroid transport is diminished as a result of the changes in the protein.

The change in steroid inhibitory activity associated with the Vinca-related mutations, shown in Fig. 8, presumably reflects the ability of 5βPodo to cause and increase in drug uptake at lower steroid concentrations in the MSVbC cell lines than in MS23 cells. This behavior can be tested by taking advantage of the fact that the A245D mutation, expressed in the MSVbC-32 cell line, does not cause a change in the resistance to daunomycin (Table 2). Flow cytometry was used to measure the daunomycin content of W7TB, MS23, and MSVbC-32 cells that had been incubated with daunomycin and either 5βPodo or dexamethasone. Although dexamethasone is a substrate for transport by the mdr1b protein, it does not normally exhibit inhibitory activity, even at concentrations as high as 10 μM (Gruol and Bourgeois, 1997). Figure 9 illustrates the results of these studies. All of the values reflecting daunomycin accumulation in the MS23 and MVbC-32 cells have been normalized to the corresponding value obtained with the W7TB line (no P-gp expression). Thus, in the left, the MS23 cells without inhibitor are shown to accumulate only 28% of the daunomycin that was found in the W7TB cells. 5βPodo had little effect at 0.1 μM, but at 1 μM, it nearly doubled the daunomycin content. In comparison, 5βPodo was found to have a much greater capacity to promote daunomycin accumulation in the MSVbC-32 cells. At 0.1 μM 5βPodo, the daunomycin levels were nearly 60% that of W7TB, and at 1 μM the daunomycin levels were the same in the W7TB and MSVbC-32 cells. As expected from previous results, dexamethasone displayed little, if any, effect on the daunomycin accumulation in the MS23 cells expressing the normal protein. With the MSVbC-32 cells, on the other hand, dexamethasone was able to cause a modest reversal of daunomycin exclusion at 1 μM and a complete reversal at 10 μM. Therefore, dexamethasone has gained the

TABLE 2
Summary of the changes in the drug resistance profiles for variants expressing the vinca-related mutations
The effects of the listed mutations on the drug resistance profiles were determined as described for Table 1.

Cell line	Paclitaxel	Vincristine	Daunomycin	Dexamethasone	Puromycin	Colchicine	Mutation
MSVbC-2	▾▾	▾▾	+	▾▾	N.C.	N.C.	K241N
MSVbC-9	▾▾▾	▾▾	N.C.	▾▾	N.C.	N.C.	K241T
MSVbC-13	▾▾▾	▾▾	N.C.	▾	N.C.	N.C.	F238S
MSVbC-32	▾▾▾	▾▾▾	N.C.	▾▾	N.C.	+++	A245D

N.C., no change; +, NCR increase ≥50%; ++++, ≥200%; ▾, NCR decrease ≥ 33%; ▾▾, ≥67%; ▾▾▾, ≥100%.

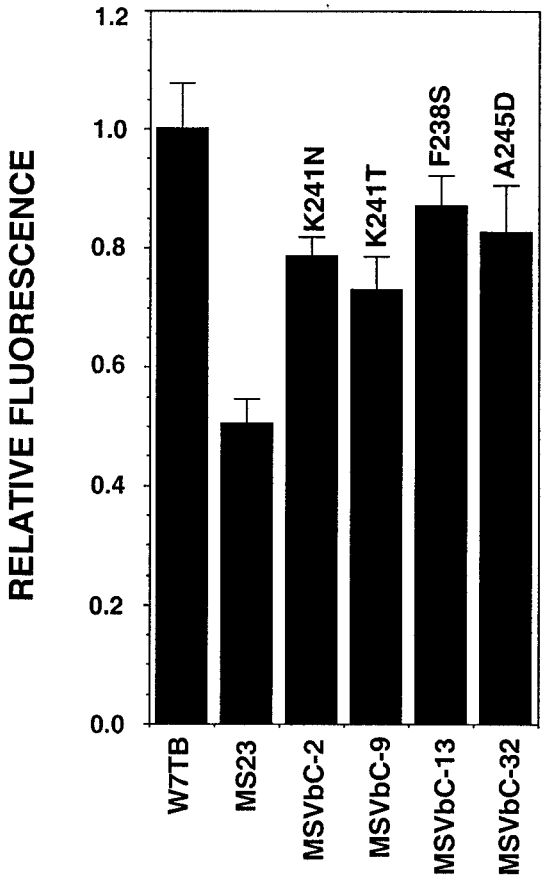

Fig. 7. The effects of the Vinca-related mutations on the intracellular accumulation of BODIPY-vinblastine. Cell cultures of the indicated cell lines were incubated with 15 nM BV for 1 h at 37°C in Dulbecco's modified Eagle's medium. After removing the free BV by centrifugation and resuspension in chilled (0°C) phosphate-buffered saline, the samples were subjected to flow cytometry analysis as described under *Materials and Methods*. The levels of BV accumulation were normalized to the values obtained with W7TB (no P-gp expression). The data represent the average of two separate experiments

capacity to inhibit the mutated P-gp expressed in MSVbC-32, possibly because of the reduction in the protein's capacity to transport it.

Discussion

The underlying assumption for the approach described here is that P-glycoproteins contain circumscribed regions that bind different classes of hydrophobic molecules through interactions with specific chemical features within each type of molecule. Depending upon the number and nature of these interactions, drugs may serve as substrates for, or inhibitors of, transport. This distinction may not be absolute but is supported by the work of Scala et al. (1997) who have shown that P-gp substrates and antagonists tend to cluster within one group or another. For corticosteroids, the presence of an 11β-hydroxyl group (see Fig. 1) is a major determining factor in conveying a capacity to the compound to serve as a substrate for transport (Bourgeois et al., 1993). Carbonyl groups associated with the C-3 and C-20 atoms, along with a 17α-hydroxyl group, all act to promote the inhibitory activity of pregnanes (Vo and Gruol, 1999). Thus, these moieties serve to promote the initial interaction with the mdr1b P-glycoprotein. It follows that mutations that disrupt the recognition of these groups would interfere with the ability of both the inhibitor and substrate forms to interact productively with the protein. This line of reasoning serves as the basis for the two criteria that we have used to classify the amino acids as contributing to a drug interaction domain. Their mutation must cause a reduction in the capacity of both the inhibitor form to reverse drug resistance and the toxic form to be transported.

The structure(s) that bind the different classes of drugs may be formed by one or more α-helical segments of the protein that traverse the plasma membrane. Such a drug binding region, involving TMS-4 to -6 and TMS-10 to -12 has been proposed by Loo and Clarke (2000) based upon protein cross-linking experiments. However, a major question still remains. Where, relative to one another, do different classes

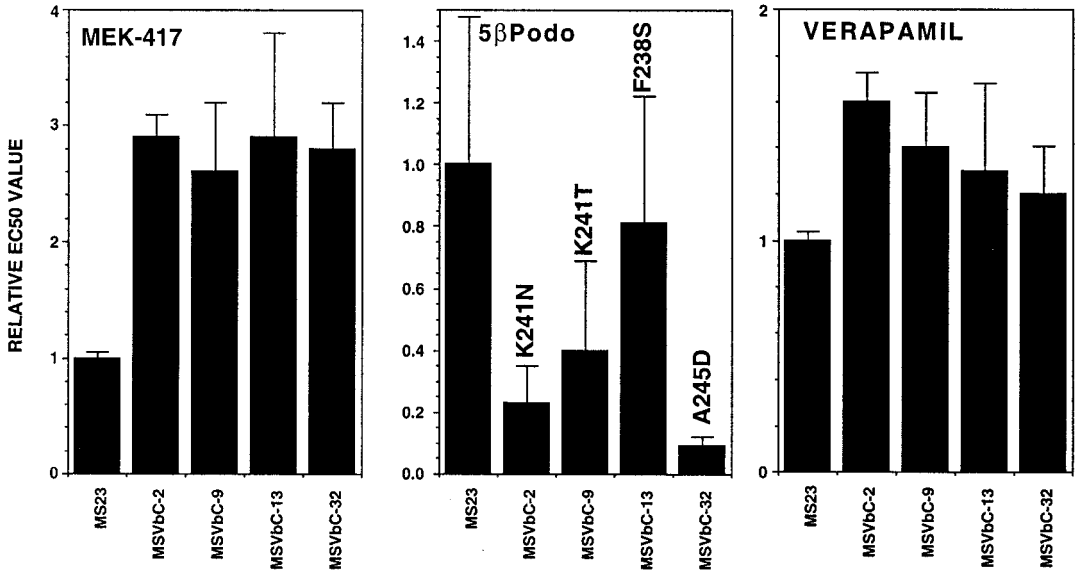

Fig. 8. The effects of the Vinca-related mutations on the inhibitory activities of MEK-417, 5βPodo, and verapamil. The relative EC_{50} values for MEK-417, 5βPodo, and verapamil were determined for the variants expressing Vinca-related mutations as in Fig. 3B. In this instance, the cells were incubated with a fixed concentration of puromycin (5 μM)

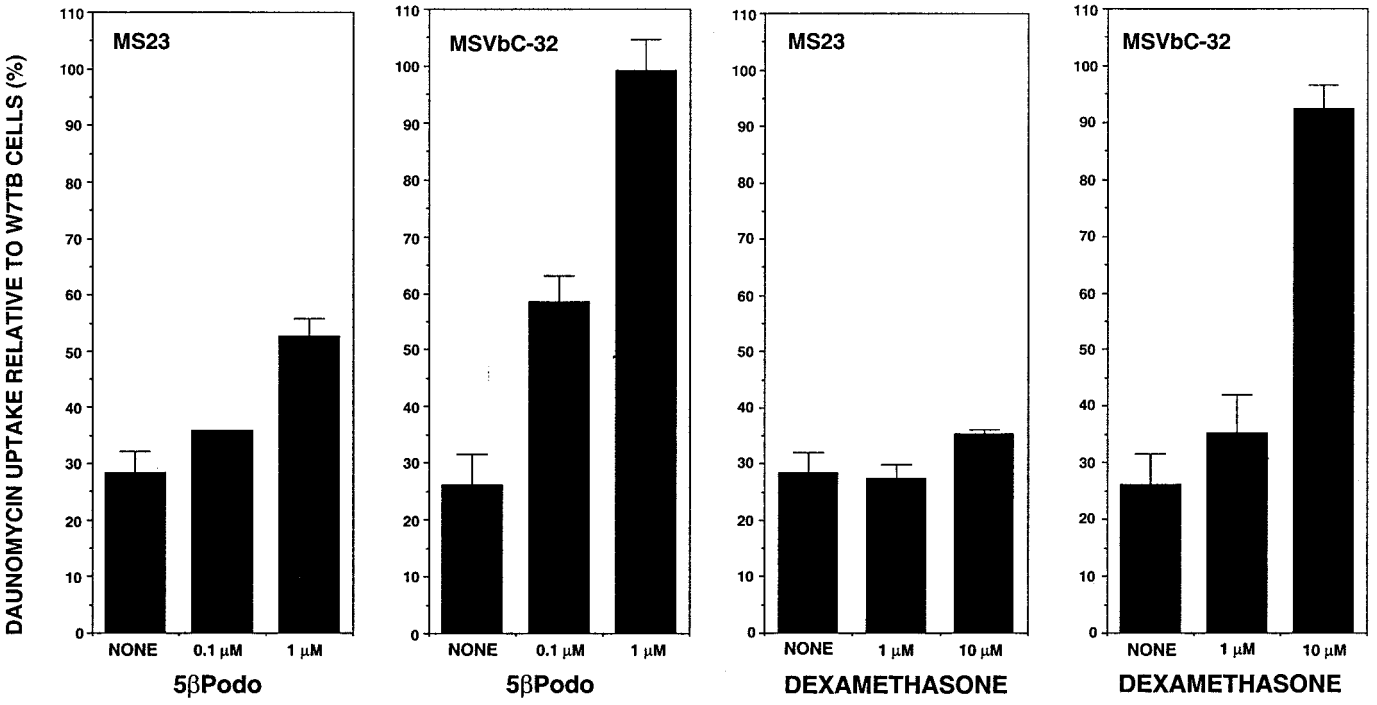

Fig. 9. The effects of 5βPodo and dexamethasone on the daunomycin accumulation in MS23 and MSVbC-32 cells. Cell cultures were incubated with 1 μM daunomycin for 1 h in the presence of the indicated concentrations of either 5βPodo or dexamethasone. The samples were collected by centrifugation, resuspended in cold phosphate-buffered saline, and subjected to flow cytometry analysis as described under *Materials and Methods* and in Fig. 7. The levels of daunomycin accumulation in the MS23 and MSVbC-32 cells were normalized relative to the values obtained with W7TB (no P-glycoprotein expression) cells.

of compounds interact within the binding region? One example of a structure that can interact with a variety of hydrophobic compounds is provided by the *Staphylococcus aureus* multidrug binding protein QacR. The QacR protein is a transcription repressor that binds a variety of mono and bivalent cationic lipophilic drugs. This includes two drugs that are substrates for P-glycoproteins, rhodamine 6G and crystal violet (Gruol and Bourgeois, 1997). X-ray crystallographic studies by Schumacher et al. (2001) have revealed that the structure of this 23-kDa protein is almost entirely α-helical. The results have also demonstrated the existence of several distinct but linked binding sites within one multifaceted drug-binding pocket. The same may be true for P-gp. Figure 2 illustrates that the P-gp mutations that alter the ability of steroids to act as either substrates or inhibitors are confined to a small portion of the protein within TMS-4 to -6. The location of the mutations also supports the possibility that steroids interact with the protein within the region that spans the cytoplasmic leaflet of the plasma membrane. However, the steroid interaction domain does not seem to overlap that of the taxane interaction domain (Gruol et al., 2001).

There are a number of observations, related to the identification of the 10 steroid-related mutations, that support the existence of a structure-specific binding site localized within the first half of the protein. Foremost is the fact that no mutations satisfying the dual criteria of causing reductions in both dexamethasone resistance and 5βPodo inhibition have been found elsewhere in the protein. This includes the results of selections carried out with the vinblastine-based inhibitor MEK-417, a taxane-based inhibitor, tRA-96023 (Gruol et al., 2001), and verapamil (D. Gruol, unpublished results). In addition, more than a dozen independent selections using a steroid as the selective inhibitor yielded variants expressing either the W231L or the W231C mutation. Multiple mutations were also found at the A301 residue (A301V, A301T, A301D), although much less frequently than the changes at W231. Thus, there has been repeated indication that these two amino acids make an important contribution to the protein's interaction with steroids. It is also worth noting that all three of the mutated amino acids in TMS-4 (S228, A229, and W231) are clustered within a four-amino acid segment. Moreover, the steroids described here are small enough to be entirely contained within the inner leaflet of the plasma membrane.

The P-gp inhibitor RU486 provides a valuable test for ligand specificity of the steroid interaction domain defined by the 10 steroid-related mutations. Previous studies demonstrated that both the 17α-hydroxyl and 20-carbonyl groups of 5βPodo make an important contribution to its inhibitory activity (Vo and Gruol, 1999). RU486 lacks both of these features (see Fig. 1). The 17-hydroxyl group of RU486 is in a β orientation and RU486 has a 17α-propynylestra group instead of the 17β-ethyl group of pregnanes. Thus, the failure of the 10 steroid-related mutations to cause a significant reduction in RU486 inhibitory activity suggests that this steroid inhibitor interacts at another site within the protein. Consistent with this interpretation is the fact that RU486 inhibits both the mdr1a and mdr1b P-gp with nearly equal effectiveness (data not shown). Conversely, the mdr1a protein is only poorly inhibited by 5βPodo (Gruol et al., 1999). This is not surprising because within 30 to 36 amino acids of TMS-4 to -6 that may traverse the inner leaflet, there are at least 14 amino acid differences between the two related forms of P-gp.

We have identified and characterized four mutations that indicate the existence of a distinct Vinca interaction domain. These mutations are clustered within a protein segment proximal to TMS-4 and are thought to be within the cytoplasmic compartment. Although there is no indication that the steroid and Vinca domains overlap, three of the four mutations cause a unique phenotypic change in the substrate and inhibitory activities of steroids. The mutations expressed in the MSVbC-2, -9 and -32 variants cause a significant loss in dexamethasone resistance (−80, −77, and −67% respectively), which is accompanied by an increase in 5βPodo inhibitory activity. Moreover, dexamethasone can function as an inhibitor in these forms of the protein. The retention and enhancement of steroid inhibitory activity strongly suggests that the Vinca-related mutations do not significantly interrupt the initial interaction between the steroids and the protein. Therefore, the coincident reduction in dexamethasone resistance is likely to reflect that the mutations interfere with a subsequent step, activation of the transport process. Based upon this line of reasoning, we propose that one of the consequences of a steroid substrate interacting with TMS-4 is the initiation of a structural change that is propagated into the cytoplasmic segment immediately distal to it. This, in turn, may influence the level of ATPase activity that drives drug transport.

There is one other example of a mutation located outside of the steroid interaction domain causing enhanced 5βPodo inhibitory activity (Gruol et al., 2001). Experiments carried out to define a taxane interaction domain led to the isolation of the 24TCTP-6 cell line. This variant was isolated by sequential selections, the first of which led to the isolation of a cell line, MSTC-24, expressing the P-gp mutation L868W. A second selection, using the MSTC-24 cells, resulted in the isolation of the 24TCTP-6 cell line, which expresses P-gp containing the mutations L868W and N988D. The acquisition of the N988D mutation, located in TMS-12, caused an increase in 5βPodo inhibitory activity (relative EC_{50} value of 0.5). In contrast to the Vinca-related mutations, acquisition of the N988D mutation was also coincident with a large increase in dexamethasone resistance. Thus, the mutation in TMS-12 seems to "allow" an enhanced interaction between steroids and the protein, possibly by removing a suppressive modulating effect. This behavior is likely to reflect a direct interplay between TMS-4 and TMS-12. Loo and Clarke (2000) demonstrated that, for the human P-gp, the region containing the S993 residue (S991 in the mouse mdr1b P-gp) is located very near W232 (W231 in the mouse mdr1b P-gp). This was accomplished through cross-linking experiments with the oxidative agent copper phenanthrolene, which is a zero-length cross-linking agent. As described above, we have identified W231 as playing an important role in the interactions between steroids and the protein. Thus, our results provide an indication of the functional significance associated with the physical proximity of limited sections of TMS-4 and TMS-12.

The discovery of Vinca-related mutations located within a proposed cytoplasmic portion of the protein was unexpected. Drugs are thought to be transported from within the lipid bilayer. However, vinblastine has two separate multiring components, each larger than 5βPodo. During its passage into the cell, vinblastine may partially reside within both the membrane and cytoplasmic compartments for a significant period of time. The advantage of such a possibility would be a potential for additional drug/protein contacts, which could also expand the total repertoire of drugs that are P-gp substrates. Conversely, the P-gp may interact with the Vinca drugs that are entirely within the cytoplasmic compartment. It is worth noting that in vitro saturation binding of vinblastine to P-gp-containing membranes has been demonstrated (Martin et al., 2000). The observed K_d for the binding was 10 nM. This behavior satisfies the requirement for high affinity binding, which would be necessary for the protein to interact effectively with the relatively low concentrations of the drug that may exist in the cytoplasm.

Acknowledgments

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