Chemosensitizing Steroids: Glucocorticoid Receptor Agonists Capable of Inhibiting P-Glycoprotein Function

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ABSTRACT

P-glycoprotein expression in lymphoid malignancies has the potential to compromise the efficacy of many therapeutic regimens using anthracyclines, glucocorticoids, and Vinca alkaloids. All three classes of drugs are transported by P-glycoproteins. We have explored the possibility that modified steroids could serve a dual purpose, as glucocorticoid receptor agonists and P-glycoprotein inhibitors. Substitution of such steroids for those currently in use would help to overcome the selective advantage held by cells expressing P-glycoproteins. 17-Deoxydexamethasone and dichloro-11(3)-fluoro-16a-methyl-3,20-dione 21-(4-dimethylamino benzolate); SA47, 1,4-pregnadiene-9a-fluoro-16a-methyl-11b,21-diol-3,20-dione; DiDOD, 1,4-pregnadiene-9a-fluoro-16a-methyl-11b,21-diol-3,20-dione; DiDOP, 1,4-pregnadiene-9a-fluoro-16a-methyl-11b,21-diol-3,20-dione (21-4-dimethylamino benzolate); SA47, 1,4-pregnadiene-9a, 11b-dichloro-17a,21-diol-3,20-dione 21-4-dimethylamino benzolate).

INTRODUCTION

P-glycoproteins are plasma membrane-spanning proteins capable of transporting a wide variety of hydrophobic drugs out of cells (1–3). This property has the potential of influencing the outcome of chemotherapy, because a significant number of anticancer drugs are substrates for transport by these proteins (4, 5). However, although the transport of xenobiotics has been studied extensively, relatively little is known about P-glycoprotein transport of endogenous compounds. Recently, we and others obtained evidence of specific glucocorticoid hormone transport by the human and mouse MDR1 P-glycoproteins (6–9). The relatively high level of P-glycoprotein expression in the adrenal glands (10), the site of glucocorticoid synthesis, also suggests a physiological role for the protein in glucocorticoid transport. Moreover, P-glycoprotein expression in lymphoid cells can act to protect them from glucocorticoid-induced apoptosis (9). This phenomenon may take on additional significance in lymphomas and leukemias, the chemotherapy of which often includes glucocorticoids (dexamethasone or prednisolone), as well as other P-glycoprotein substrates (11–15).

Not all steroids that interact with P-glycoproteins are substrates for transport. Progesterone can bind to and photoaffinity-label P-glycoproteins, as well as inhibit transport of other drugs (16–18). Progesterone is not, however, transported by the mouse or human P-glycoproteins (7, 9, 17). The glucocorticoid cortisol, on the other hand, is efficiently transported (7, 9). The structural difference between the two compounds is the presence of three hydroxy groups associated with the 11, 17, and 21 carbon atoms of cortisol, but not progesterone. Other steroids lacking either the 11b- or 17a-hydroxy groups (cortisolone and corticosterone) are less efficiently transported than cortisol (9). Thus, the 11b- and 17a-hydroxy groups, which play a very significant role in determining whether a steroid can function as a glucocorticoid, also act to distinguish it as a potential substrate for P-glycoproteins.

The progestin/gluocorticoid inhibitor RU38486 (RU486) can also cause a reversal of MDR by promoting increased drug uptake in cells expressing the murine and human P-glycoproteins (19, 20). Similar to progesterone, RU486 does not appear to be a substrate for transport by P-glycoproteins (21). A direct comparison between RU486 and progesterone has shown that RU486 is at least 2 times more effective at inhibiting rhodamine 123 efflux in murine thymoma cells expressing P-glycoproteins (19). There are a number of structural differences between RU486 and progesterone that could contribute to the relative disparity in their chemosensitizing capacities. In the work presented here, we combine steroid structural features that contribute to chemosensitizing capacity with those that promote glucocorticoid receptor agonist activity. The goal was to explore the feasibility of producing a new class of steroids with a dual activity, chemosensitizing glucocorticoids. Such compounds could potentially serve as beneficial substitutes for the steroids dexamethasone and prednisolone, which are currently being used in the treatment of lymphoid malignancies.

MATERIALS AND METHODS

Cell Culture. WEHI-7 is a thymoma cell line obtained from a female BALB/c mouse after exposure to X-irradiation (22). W7TH is a derivative of WEHI-7, which is resistant to bromodeoxyuridine, a marker that is unrelated to multidrug resistance. S7CD-5 is a derivative of W7TH that was selected through a series of steps for resistance to the combination of dexamethasone and colchicine (23). S7CD-5 cells overexpress the murine mdr1 gene (9). The S7dexr-1 cell line was derived from S7CD-5 through a process involving mutagenesis with N-methyl-N’-nitro-N-nitrosoguanidine (24) and selection by growth in 5 × 10⁻⁷ M triamcinolone acetonide. The glucocorticoid receptors in S7dexr-1 cells have lost approximately 90% of their capacity to bind steroids. Thus, these cells are completely resistant to glucocorticoid-mediated apoptosis. The S7dexr-1 cells do not significantly differ from S7CD-5 cells in their resistance to drugs such as colchicine, daunomycin, and puromycin. All of the mouse cell lines were grown in suspension in DMEM containing 10% fetal bovine serum. The incubator was maintained at 37°C and had a humidified atmosphere of 13% CO₂ and 87% air. Two human leukemic lymphoblastic lines, developed by Beck et al. (25), were also used. The CEM/VLB100 line was derived from CEM cells by a series of selections involving vinblastine sulfate. CEM/VLB100 cells overexpress the human MDR1 gene and are not sensitive to glucocorticoid-induced apoptosis. The human cell lines were grown in RPMI 1640 containing 10% fetal bovine serum. The incubator was maintained at 37°C and had an atmosphere of 5% CO₂ and 95% air. The number of living cells in the cultures was determined by the trypan blue exclusion technique.

Quantification of Drug Effects on Cellular Proliferation. The effect of drugs on cell proliferation was measured as described previously (26). Briefly, cell cultures were set up (5 × 10⁴ cells/ml) in varied concentrations of drugs and incubated for 5 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 LD₅₀ is defined as the concentration of drug that produces a relative turbidity of 50%. The relative degree of P-glycoprotein-dependent drug resistance was evaluated by calculating LD₅₀ ratios. The LD₅₀ ratio for a given drug is defined as the LD₅₀ obtained from cells expressing P-glycoprotein divided by the LD₅₀ obtained from a cognate cell line not expressing the P-glycoprotein. In the cases presented in this report, the LD₅₀ obtained with S7CD-5 cells were divided by the LD₅₀ obtained with W7TB cells (Tables 1 and 2).

The relative activity of nontoxic drugs to reverse P-glycoprotein-dependent drug resistance was evaluated as follows: multidrug resistant cells (S7dexr) or CEM/VLB₉₀ were grown at a fixed concentration of toxic drugs to which they would normally be resistant on their P-glycoprotein expression (20 μM puromycin for S7dexr cells and 30 nM vinblastine for CEM/VLB₉₀ cells). Increasing concentrations of a P-glycoprotein-inhibiting compound were included in the culture medium, and the relative turbidity values of the cultures were evaluated after 5 days. The efficiency of a chemosensitizer is expressed by an ED₅₀ defined as the concentration of chemosensitizer that reduces the relative turbidity to 50%.

The toxic drug concentrations used to evaluate the ED₅₀ were chosen to lie between the LD₅₀ obtained from cells expressing P-glycoprotein and the LD₅₀ for cells that do not express P-glycoprotein (i.e., W7TB versus S7dexr-1; CEM versus CEM/VLB₉₀). For example, the puromycin LD₅₀ for W7TB and S7dexr cells were 1.5 and 60 μM, respectively. Thus, there was a total difference of 58.5 μM in LD₅₀ concentrations between the two cell lines. An LD₅₀ of 20 μM represents a 40 μM decrease in resistance (60—20 μM) and a 68% (40 μM/58.5 μM × 100%) reversal of the total resistance in the MDR cell line. A similar calculation was made to determine that an LD₅₀ of 30 nM represented an 80—85% reversal of vinblastine resistance in CEM/VLB₉₀ cells. Therefore, the ED₅₀s are those concentrations of chemosensitizer that reverse puromycin resistance 65—70% in S7dexr-1 cells, and vinblastine resistance 80—85% in CEM/VLB₉₀ cells.

Daunomycin Accumulation. The intracellular daunomycin content of cells was measured using flow cytometry and the inherent fluorescence of the drug. Five separate S7dexr-1 cell cultures were incubated with daunomycin (5 μM) for 35 min at 37°C. The individual samples also contained either no addition or 5 μM of one of the following: verapamil, RU486, RU42764, or progesterone. At the end of the incubation period, the cells were washed free of drugs using cold (0°C) PBS. The amount of daunomycin remaining associated with the cells was measured by flow cytometry using a Becton Dickinson FACScan analyzer (FL1). Ten thousand events were evaluated per measurement. Laser excitation was at 15 mW from a laser at 488 nm. The geometric mean values of each distribution were calculated and normalized to the value obtained in the absence of any P-glycoprotein inhibitor (no addition).

Chemicals. The following compounds were obtained from Sigma Chemical Co. (St. Louis, MO): crystal violet, daunomycin, dexamethasone, N-methyl-N'-nitro-N-nitrosoguanidine, prednisolone, progesterone, puromycin, triamcinolone acetonide, and vinblastine sulfate. The following steroids were obtained from Steraloids Inc. (Wilton, NH): DOD; DOP; DiDOD; 4 pregnen-11β-ol-3,20-dione; 1,4 pregnadiene 3,20-dione; and dichlorisone. The following compounds were custom-synthesized by Zielsinski Laboratories (San Diego, CA): DiDOD; SA450; and SA47. RU486 and RU42764 were generously provided by Roussel Uclaf (Romainville, France).

RESULTS

Glucocorticoid Agonists as Substrates for Transport by P-Glycoproteins. Our previous studies demonstrated that a variety of similar steroids were transported by the MDR1 P-glycoprotein, but with significantly differing efficiencies (9). This observation suggested that protein recognition of specific steroid structural features could be involved in the binding or transport steps that result in steroid efflux. For instance, the increased resistance to cortisol in glucocorticoid-sensitive mdr1-expressing cells was more than 10 times greater than the increased resistance to corticosterone. The only structural difference between the two compounds is the presence of a 17α-hydroxy group in cortisol. Further evidence of a structural influence on efflux efficiency was seen by comparing the results with prednisolone (see the structure in Fig. 1) and cortisol. Prednisolone, containing two carbon-carbon double bonds in its A-ring, was transported with an apparent efficiency that was 2 times that of cortisol, which has only a single carbon-carbon double bond in its A-ring.

Fig. 2A illustrates the shift in prednisolone resistance observed between W7TB cells, expressing no MDR1 P-glycoprotein, and a derivative of W7TB (S7CD-5) that expresses the transport protein. The ~60-fold shift in the prednisolone concentration needed to cause apoptosis in the S7CD-5 cells, relative to W7TB cells, indicates that prednisolone can be efficiently transported by the MDR1 P-glycoprotein. In comparison, Fig. 2B shows that there was little increased resistance exhibited by the S7CD-5 cells to an analogue of prednisolone lacking the 17α- and 21-hydroxy groups (DiDOD; see Fig. 1 for the structure). Table 1 summarizes the results of a series of similar studies involving prednisolone and several related steroids. Fig. 1 illustrates the structures of six of the compounds that were used. The LD₅₀ ratios (defined in "Materials and Methods") provide a quantification of the relative resistance of S7CD-5 cells for a given steroid. The comparisons between prednisolone versus DOP and dexamethasone versus DOD confirm the observation that we had made with prednisolone (9) that we had made with prednisolone (9). The basis of this resistance is a loss in glucocorticoid receptor function. Thus, glucocorticoids no longer act as toxic drugs but can serve as P-glycoprotein inhibitors. In comparison, Fig. 2B shows that there was little increased resistance exhibited by the S7CD-5 cells to an analogue of prednisolone lacking the 17α- and 21-hydroxy groups (DiDOD and DiDOP) were progressively reduced. DiDOP yielded only minimal evidence of being transported by the P-glycoprotein. Another analogue of prednisolone, 17α progesterone, does not contain any hydroxy groups and showed no evidence of being transported. Thus, the presence of hydroxy groups on glucocorticoid agonists contributes to the ability of P-glycoprotein expression to confer resistance to steroid-induced apoptosis. (It should be noted that 17α progesterone differs from progesterone in that it contains two double bonds in its A-ring, rather than one.)

Glucocorticoid Agonists as Chemosensitizers. Progesterone is not transported by the MDR1 P-glycoprotein, but has been shown to be an effective inhibitor of the ability of the protein to transport other drugs (17). We found that this property is true of 17α progesterone as well (data not shown). However, neither of these steroids contains hydroxy groups. Dexamethasone, prednisolone, and their analogues offer an opportunity to look further at the relationship between steroid structural features and the ability to confer resistance to steroid-induced apoptosis. The basis of this resistance is a loss in glucocorticoid receptor function. Thus, glucocorticoids no longer act as toxic drugs.

Table 1 Relative shift in LD₅₀ for steroids related to dexamethasone and prednisolone

<table>
<thead>
<tr>
<th>Steroid</th>
<th>LD₅₀ ratio</th>
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<tbody>
<tr>
<td>Prednisolone</td>
<td>60 ± 3.0 (n = 3)</td>
</tr>
<tr>
<td>DOP</td>
<td>3.3 ± 1.5 (n = 4)</td>
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<tr>
<td>DiDOP</td>
<td>1.7 ± 0.5 (n = 5)</td>
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<tr>
<td>17α Progesterone</td>
<td>1.1 ± 0.06 (n = 5)</td>
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<tr>
<td>Dexamethasone</td>
<td>42 ± 5.5 (n = 4)</td>
</tr>
<tr>
<td>DOD</td>
<td>6.4 ± 0.5 (n = 3)</td>
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<tr>
<td>DiDOD</td>
<td>2.7 ± 0.5 (n = 4)</td>
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The values represent the ratio of LD₅₀₉₀ obtained with the listed steroids using the W7TB and S7CD-5 cells as illustrated in Fig. 2. A discussion of the calculation is included in "Materials and Methods." The LD₅₀ ratios represent the relative capacity of the P-glycoprotein expressed in the S7CD-5 cells to reduce the intracellular concentration of steroid (reflected by the capacity to induce apoptosis). Values significantly >1.0 are indicative of P-glycoprotein-dependent steroid transport.
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Fig. 1. Structures illustrative of the steroids used in Table 1 and Fig. 3.

in these cells. Relative resistance to other drugs, such as puromycin, remains unchanged.

Fig. 3 shows the results of using a series of steroids to reverse the puromycin resistance ~67% in S7dex'-1 cells. This was accomplished by exposing the cells to a constant concentration of puromycin (20 μM) while varying the concentration of steroids. Fig. 3A shows the effects of increasing concentrations of prednisolone, DOP, and DiDOP. Thus, in both series, each successive steroid differs from the previous one by a single hydroxy group. Under these conditions, neither prednisolone nor dexamethasone had the capacity to act as a chemosensitizer. The absence of a 17α-hydroxy group, however, imparted a capacity to reverse the resistance to puromycin. DOD and DiDOD each exhibited the capacity to increase drug toxicity in these cells. The absence of both the 17α- and 21-hydroxy groups produced even greater chemosensitizing capacity. The dexamethasone analogue DiDOD (ED50 = 0.63 μM) was significantly more effective than the prednisolone analogue DiDOP (ED50 = 1.7 μM). By way of comparison, progesterone and 1Δ progesterone were found to have ED50 of 1.7 and 2.1 μM in this assay, respectively (data not shown). The results indicate that a progression toward greater hydrophobicity and lower capacity to be efficiently transported produced an increased chemosensitizing capacity. The results, particularly with the dexamethasone derivative DiDOD, also demonstrate that there may be effective glucocorticoid agonists that could serve as potent inhibitors of P-glycoprotein function.

Our earlier studies demonstrated that the antiglucocorticoid/progestin RU486 has the capacity to reverse drug resistance in cells expressing the mdr1 gene (19). We found RU486 to be more than 2 times as efficient as progesterone at inhibiting P-glycoprotein-dependent rhodamine 123 efflux from cells. Fig. 4A shows the results of a set of additional studies and illustrates the effects of RU486, RU42764, progesterone, and verapamil on the ability of S7dex'-1 cells to accumulate daunomycin. Fig. 4B illustrates the structures of the four P-glycoprotein inhibitors. RU486 and RU42764 differ from each other by the substituents associated with their 11 carbon atoms. The data presented in Fig. 4A represent the relative increase in cellular daunomycin fluorescence due to P-glycoprotein inhibition by each drug. Progesterone caused an increase of 0.8-fold, whereas RU486 increased the daunomycin content by 1.8-fold. These data are in agreement with our earlier results (19). RU486 was slightly less effective than verapamil, whereas RU42764 was virtually identical to progesterone in its effectiveness. A comparison of the results obtained with RU486 and RU42764 indicates that the DMAP group of RU486 may make a contribution to the chemosensitizing capacity of the steroid.

We have extended our studies with RU486 to evaluate its capacity to inhibit the human MDR1 P-glycoprotein expressed in two leukemic cell lines (25). Fig. 5A illustrates the sensitivity of the CEM (no P-glycoprotein expression) and CEM/VLB100 cell lines to vinblastine. CEM/VLB100 overexpresses the P-glycoprotein and, in the example shown here, exhibited a 120-fold increase in vinblastine resistance. Addition of 5 μM RU486 to the cultures caused a 97% reversal of vinblastine resistance, whereas the same concentration of RU42764 caused only a 67% reversal. These observations confirm that the difference in chemosensitizing capacities observed between these two drugs with the mouse P-glycoprotein also exists with the human protein. Verapamil appeared slightly more potent than RU486 in this assay, whereas RU42764 and progesterone (data not shown) were very similar. Fig. 5B illustrates that neither RU486 nor RU42764 had a significant effect on the vinblastine sensitivity of the CEM cell line. Verapamil, on the other hand, increased the sensitivity of the cells >2-fold in a manner that was presumably independent of P-glycoprotein expression. Thus, the reversal of vinblastine resistance observed with RU486 and RU42764 in Fig. 5A did not appear to be due to a nonspecific sensitization of the cells.

The comparison of chemosensitizing capacities of RU486 and RU42764 shown in Figs. 4 and 5 supports the proposition that the DMAP group of RU486 can enhance the capacity of steroids to inhibit P-glycoprotein drug transport. In an additional effort to investigate the
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Fig. 2. Comparisons of prednisolone and DiDOP sensitivities in W7TB and S7CD-5 cells. A, cell cultures, initially containing $5 \times 10^6$ cells/ml, were incubated in medium containing the indicated concentrations of the steroid prednisolone 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 steroid. Each point is the average of two determinations. B, the same protocol as in A, but using the steroid DiDOP. ▲, W7TB cells; ▲, S7CD-5 cells.

Putative interaction of DMAP groups with P-glycoproteins, we have evaluated the effects of the compound crystal violet on W7TB and S7CD-5 cells. Fig. 6A illustrates that crystal violet is composed of DMAP groups linked to a single carbon atom. The structure of the compound allows a resonance of the double bond associated with the central carbon atom. This situation results in a partial positive charge on each of the nitrogen atoms and a more planar molecule, overall. Moreover, crystal violet is toxic at submicromolar levels, which provides the means to test whether it is a substrate for transport by P-glycoproteins. The data shown in Fig. 6B demonstrate that the S7CD-5 cells were more resistant to crystal violet than W7TB cells. The $LD_{50}$ ratio was 4.2. A comparable result was also obtained with CEM and CEM/VLB$_{100}$ cells (data not shown). If crystal violet is transported by P-glycoproteins, P-glycoprotein inhibitors will reverse the resistance conveyed by the compound's efflux. Fig. 7 illustrates the relative capacities of RU486, RU42764, and verapamil to reverse crystal violet resistance in CEM/VLB$_{100}$ cells. The experiment was carried out in a manner similar to that shown in Fig. 3. CEM/VLB$_{100}$ cells were grown in a concentration of crystal violet to which they are resistant, but which is toxic to CEM cells that do not express the P-glycoprotein. The results demonstrate that both RU486 and verapamil effectively reversed crystal violet resistance at concentrations below 5 μM. RU42764 was ineffective at the concentrations used in this assay. Studies with cells (S7dexr-1) expressing the mouse MDR1 P-glycoprotein produced similar results (data not shown).

In contrast to RU486, RU42764 is both a glucocorticoid agonist and a P-glycoprotein inhibitor. However, our preliminary experiments have shown that RU42764, as well as DiDOD and DiDOP, are not very potent inhibitors of the human MDR1 P-glycoprotein. Consequently, we have explored the possibility of adding a DMAP group to other glucocorticoid agonists through the 21-carbon atom of the steroids. Two glucocorticoids were chosen to be modified: DOD and dichlorisone. Fig. 8 illustrates the structures of both steroids and their dimethylamino benzoate derivatives. Dichlorisone contains a 17α-hydroxy group, but has a chlorine atom instead of a 11β-hydroxy group. This feature results in a compound that is a glucocorticoid agonist, but is less likely to be transported by P-glycoproteins. SA450 is the DOD derivative, and SA47 is the dichlorisone derivative. Fig. 9 depicts the relative abilities of verapamil, SA450, and SA47 to reverse vinblastine resistance in CEM/VLB$_{100}$ cells. The cells were grown in increasing concentrations of vinblastine while the concentration of each inhibitor used was held constant (2 μM). Under these conditions, all three of the compounds had a nearly identical effect and produced a >90% reversal of resistance. When the inhibitors' concentration was reduced to 1 μM, SA450 still produced >80% reversal (84 ± 2.1%, n = 2), whereas the degree of reversal for SA47 and verapamil were significantly less, 74 ± 7.8% (n = 2) and 75 ± 8.1% (n = 2), respectively.

Table 2 summarizes the results of a series of studies characterizing the agonist and chemosensitizing activities of the compounds listed. Agonist activities were evaluated by determining the $LD_{50}$s of the compounds obtained with W7TB cells (column 1). The results demonstrate that the addition of a dimethylamino benzoate moiety in SA47 and SA450 caused less than a 2-fold reduction (increase in the $LD_{50}$) in glucocorticoid agonist activity when compared with dichlorisone and DOD. Thus, the derivatives remained potent glucocorticoid receptor agonists. S7dexr-1 cells, which are refractory to the apoptotic effects of glucocorticoids, were used to evaluate the chemosensitizing effects of the compounds on the mouse MDR1 P-glycoprotein (column 2). This study was performed as illustrated in Fig. 3. Dichlorisone had no activity at concentrations up to 8 μM, whereas DOD had an $ED_{50}$ of approximately 3 μM. Addition of the dimethylamino benzoate group to these compounds greatly enhanced their chemosensitizing abilities and reduced the $ED_{50}$ of both steroids by more than 10-fold. Moreover, SA450 was 3 times as effective as verapamil at inhibiting P-glycoprotein-dependent puromycin resistance. SA47 and SA450 were also effective inhibitors of the human MDR1 P-glycoprotein (column 3). $ED_{50}$s were determined for CEM/VLB$_{100}$ cells grown in 30 nM vinblastine. This condition represents an 80–95% reversal of vinblastine resistance in these cells. SA450 was, again, the most potent chemosensitizer of the series. The $ED_{50}$ of SA450 was approximately two-thirds that of verapamil and one-half of that of SA47. When added to cultures of CEM cells in the absence of other drugs, neither of the two new compounds produced a great reduction in cell viability at concentrations below 40 μM. The $LD_{50}$ for SA47 was 50 μM, and the $LD_{50}$ for SA450 was ~100 μM.

The abilities of SA47 and SA450 to be transported by the murine MDR1 P-glycoprotein were also tested. $LD_{50}$ ratios were determined as described for Table 1 using the W7TB and S7CD-5 cell lines. The dichlorisone derivative SA47 gave the lowest value, 3.3 ± 0.85 (n = 4), which indicates a relatively modest capacity to serve as a substrate for transport. Because dichlorisone by itself gave very little evidence of being transported or acting as a chemosensitizer, we...
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conclude that its modification was the cause of the resulting steroid interacting more productively with P-glycoproteins. The \( \text{LD}_{50} \) ratio for SA450 was 7.1 ± 2.1 (n = 4). Thus, there was relatively little alteration in this steroid’s transport when compared with the original compound DOD (\( \text{LD}_{50} \) ratio of 6.4; see Table 1).

Discussion

In 1976, Bech-Hansen et al. (27) reported that multidrug-resistant Chinese hamster ovary cell lines were hypersensitive to a series of compounds that included the steroids 5β pregnane 3,20 diol and corticosterone. This collateral sensitivity correlated with the degree of cross-resistance to other drugs in these cell lines. The subsequent demonstration that the multidrug resistance phenotype in these cells was due to P-glycoprotein expression (28) suggested that steroids could interact with P-glycoproteins. This possibility was given further credence through a series of experiments by Band Horwitz et al. (16). They showed that progesterone, and to a lesser degree other steroids, inhibited photoaffinity labeling of P-glycoproteins by azidopine. Moreover, progesterone inhibited binding of vinblastine to microsomal membranes and reversed vinblastine resistance in murine J7.V1-1 cells. However, there is no evidence that P-glycoproteins transport progesterone (7, 9, 17). Thus, progesterone binds to and inhibits P-glycoproteins without being a substrate for transport.

Glucocorticoids, such as cortisol, dexamethasone, and prednisolone, are efficiently transported by the murine MDR1 P-glycoprotein (9). Our work has demonstrated that the ability of P-glycoproteins to reduce the intracellular concentration of steroids is related to the number and location of steroid hydroxy groups. There may be several reasons for this behavior. The presence of hydroxy groups would be expected to decrease the diffusion rate of steroids into and across the plasma membrane. Low rates of influx would act to augment the P-glycoprotein-dependent efflux and contribute to the maintenance of a reduced intracellular steroid concentration. It is also possible that steroid hydroxy groups enhance the process of steroid efflux. This possibility implies a form of structural recognition by the P-glycoprotein that results in an increased rate of steroid transport by P-glycoproteins. The fact that the 11β-hydroxy group of endogenous glucocorticoids appears necessary for efflux (9) supports this supposition. Our studies of puromycin resistance in S7dexr-1 cells provide additional support for this hypothesis. We have found that DiDOP is more effective at reversing puromycin resistance than 1Δ progesterone (data not shown). The only structural difference between the two

**Fig. 3.** Reversal of puromycin resistance in S7dexr-1 cells by dexamethasone, prednisolone, and their analogues. A series of S7dexr-1 cell cultures (5 X 10⁶ cells/ml) was grown in medium containing 20 \( \mu \)M puromycin and increasing concentrations of the indicated steroid. After 5 days, the turbidities (660 nm) of the cultures were measured, and the values were expressed relative to cultures that did not contain steroid. Each point is the average of two determinations. A, prednisolone (□) and its analogues DOP (△) and DiDOP (○). B, dexamethasone (□) and its analogues DOD (△); and DiDOD (○).

**Fig. 4.** Effects of P-glycoprotein inhibitors on the accumulation of daunomycin into S7dexr-1 cells. A, S7dexr-1 cell cultures were incubated for 35 min (37°C) in medium containing daunomycin (5 \( \mu \)M) and either no additions or 5 \( \mu \)M of one of the following: verapamil, RU42764, or progesterone. At the end of the incubation period, the cells were collected by centrifugation, washed, and suspended in cold (0°) PBS. Each sample was analyzed by flow cytometry using a Becton Dickinson FACScan analyzer to measure the fluorescence due to the internalized daunomycin. The geometric mean values of each distribution were calculated and normalized to the value obtained from cells incubated without an inhibitor. The data shown here represent the averages of two separate experiments. B, the structures of the P-glycoprotein inhibitors used to promote daunomycin accumulation into the cells.
carbon ring possessing a dimethylamino group at the para position can interact with P-glycoproteins. Depending on the nature of the molecule that contains the DMAP group, these interactions can promote interference of drug transport and/or transport of the DMAP-containing compound. Thus, RU486 inhibits drug transport without being a substrate (19, 21), crystal violet is a substrate for transport, and SA47 inhibits drug transport and is a substrate for transport. Our data demonstrate that the addition of a DMAP group can increase the chemosensitizing capacity of steroids. This was true for RU486, in which the DMAP group is associated with the 11-carbon atom, and for SA47 and SA450, in which a dimethylamino benzoate group was associated with the 21-carbon atom. The fact that an aromatic dimethylaniline group can exert its influence on P-glycoprotein function while located at different parts of the steroid suggests that the different portions of these composite molecules may act with some independence of one another. Thus, the compounds may participate in multiple-site interactions with the P-glycoprotein. There is a precedent for this type of behavior. Ayesh et al. (29) have shown that the inhibitors verapamil and tamoxifen each bind to P-glycoproteins at a site distinct from that of the other. Cyclosporin, on the other hand, interacts with both of these sites. The ability of cyclosporin to bind to more than one site may be a contributing factor in its potent chemosensitizing capacity.

The rationale for seeking chemosensitizing glucocorticoids lies in the 11β-hydroxy group of DiDOP (see Fig. 1 for the structures). Thus, by comparison, the 11β-hydroxy group of DiDOP enhanced the ability of this steroid to interact with the P-glycoprotein and interfere with puromycin transport. The presence of a second hydroxy group at the 21-carbon position (DOP) caused an increased efficiency in conveying resistance to steroid-induced apoptosis (compare DiDOP and DOP, Table 1), but a reduction in the relative ability of the steroid to serve as an inhibitor of puromycin transport (Fig. 3A). This behavior is even more pronounced in the presence of a third hydroxy group at the 17-carbon position. Thus, the 17- and 21-carbon hydroxy groups may promote the active rate of steroid efflux across the membrane while causing an overall reduction in passive diffusion within the membrane. Promotion of transport rates would also lessen the time that the steroid is bound to the P-glycoprotein and lead to a renewed ability to transport other drugs. This model is consistent with the observation that the 11β-hydroxy group-containing steroids that are transported with the greatest efficiency, dexamethasone and prednisolone, are not very effective as chemosensitizers.

The work presented in Figs. 4—7 indicates that an aromatic six-
their potential ability to suppress the emergence of P-glycoprotein-dependent drug resistance during chemotherapy. The promise of this strategy was demonstrated by the work of Futscher et al. (30). They used in vitro selections to show that inclusion of an inhibitor (verapamil) suppressed the appearance of P-glycoprotein-dependent resistance to doxorubicin in human multiple myeloma cells. A similar result had also been indicated by the work of Chen et al. (31) using MCF-7 cells. Our goal is to ask whether such a suppression can be obtained.

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Fig. 7. Reversal of crystal violet resistance in CEM/VLB100 cells by verapamil, RU486, and RU42764. Three sets of CEM/VLB100 cell cultures (5 × 10⁶ cells/ml) were incubated in medium containing 0.5 μM crystal violet and increasing concentrations of either verapamil, RU486, or RU42764. At the end of a 5-day incubation period, the turbidities of the cultures were measured and evaluated as in Fig. 3. □, Verapamil; △, RU486; △, RU42764.

Fig. 8. Structures of DOD, dichlorisone, and their derivatives, SA47 and SA450.

Fig. 9. Effects of verapamil, SA47, and SA450 on the vinblastine sensitivity of human leukemic cells. A, four sets of CEM/VLB100 cell cultures (5 × 10⁶ cells/ml) were incubated for 5 days in medium containing increasing concentrations of vinblastine. The individual sets contained either no addition or 2 μM of one of the following: verapamil, SA47, or SA450. At the end of the incubation period, the turbidities of the cultures were measured and evaluated as described for Fig. 2. The vinblastine sensitivity of CEM cells (no P-glycoprotein expression) without additions was also measured. ▲, CEM/VLB100 no addition; □, CEM/VLB100 + verapamil; △, CEM/VLB100 + SA47; △, CEM/VLB100 + SA450; □, CEM no addition.

Table 2 Summary of LD₅₀ and ED₅₀ Values

<table>
<thead>
<tr>
<th>Compound</th>
<th>WTTB Cells agnostic activity</th>
<th>S7CD-5 Cells chemosensitizer</th>
<th>CEM/VLB100 Cells chemosensitizer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD₅₀ (nM)</td>
<td>ED₅₀ (μM)</td>
<td>ED₅₀ (μM)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>ND</td>
<td>0.77 ± 0.078 (11)</td>
<td>1.1 ± 0.35 (12)</td>
</tr>
<tr>
<td>RU486</td>
<td>ND</td>
<td>1.0 ± 0.34 (9)</td>
<td>1.9 ± 0.56 (12)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>6.0± 0.0</td>
<td>&gt;8 (2)</td>
<td>ND</td>
</tr>
<tr>
<td>DOD</td>
<td>6.0 ± 2.3 (7)</td>
<td>2.8 ± 0.74 (4)</td>
<td>&gt;8 (2)</td>
</tr>
<tr>
<td>DiDOD</td>
<td>8.0 ± 1.9 (4)</td>
<td>0.58 ± 0.073 (4)</td>
<td>&gt;8 (2)</td>
</tr>
<tr>
<td>SA450</td>
<td>11.7 ± 3.2 (6)</td>
<td>0.26 ± 0.15 (5)</td>
<td>0.75 ± 0.15 (4)</td>
</tr>
<tr>
<td>SA440</td>
<td>7.5 ± 1.0 (4)</td>
<td>&gt;8 (2)</td>
<td>&gt;8 (2)</td>
</tr>
<tr>
<td>SA47</td>
<td>13.1 ± 2.9 (8)</td>
<td>0.46 ± 0.099 (4)</td>
<td>1.4 ± 0.71 (5)</td>
</tr>
</tbody>
</table>

*The data are the result of a series of studies carried out with the WTTB cell line to determine the LD₅₀ of individual glucocorticoid agonist compounds. An illustration of this type of experiment is shown in Fig. 2. ND, not determined.

*These data are the result of a series of studies carried out with the S7 dex retroil cell line grown in the presence of 20 μM puromycin to determine the ED₅₀. These are the conditions used in Fig. 3.

*These data are the result of a series of studies carried out with the human CEM/VLB100 cell line. These cells were grown in the presence of 30 nM vinblastine. The measurements to calculate the ED₅₀ were made as in Fig. 3.

*This value is taken from Bourgeois et al. (1993).
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REFERENCES

Chemosensitizing Steroids: Glucocorticoid Receptor Agonists Capable of Inhibiting P-Glycoprotein Function

Donald J. Gruol and Suzanne Bourgeois


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