Steroid Transport, Accumulation, and Antagonism of P-Glycoprotein in Multidrug-Resistant Cells

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ABSTRACT: According to multiple reports, progesterone is not transported by P-glycoprotein (Pgp), which mediates multidrug resistance through active drug efflux. However, progesterone has been shown to block Pgp-mediated efflux of other drugs. To extend these observations, and to examine the effect of modulating Pgp phosphorylation, the accumulation of progesterone and 14 other steroids in untreated and calphostin C-treated multidrug-resistant human colon carcinoma SW620 Ad300 cells was compared to the accumulation in parental SW620 cells. The accumulation of progesterone in untreated multidrug resistant cells expressing Pgp was not reduced compared to parental cells. However, the accumulation of more hydrophilic steroids was reduced by as much as 50%. Progesterone and progesterone-like compounds, however, were potent inhibitors of Pgp-mediated vinblastine efflux; increased antagonism correlated with increased steroid hydrophobicity. Treatment with calphostin C, a PKC inhibitor which decreases Pgp phosphorylation, increased progesterone efflux, modulated Pgp antagonism by steroids, and inhibited photoaffinity labeling of Pgp by progesterone. These results extend previous observations that Pgp can mediate the transport of, and be antagonized by, a variety of steroids and that these properties vary with both a steroid's hydrophobicity and the phosphorylated state of Pgp.

The observation of multidrug resistance in clinical oncology has stimulated *in-vitro* studies seeking to understand this phenomenon. P-glycoprotein (Pgp),[1] originally identified by Ling in multidrug-resistant Chinese hamster ovary cells, has been demonstrated in *in-vitro* models of drug resistance and in normal and malignant human tissues (Juliano & Ling, 1976; Bell et al., 1985; Biedler & Riehm, 1970; Chin et al., 1990; Endicott & Ling, 1989; Van der Bliek & Borst, 1989; Fuqua et al., 1987; Grogan et al., 1990; Hsu et al., 1990; Pastan & Gottesman, 1987; Roy & Horwitz, 1985). These observations have heightened interest in the function of this protein in normal tissues and its role in clinical drug resistance.

P-glycoprotein is a 170-kilodalton membrane phosphoglycoprotein whose endogenous function is yet unknown, but which can serve as an ATP-dependent drug efflux pump, transporting anticancer drugs out of cells (Endicott & Ling, 1989; Van der Bliek & Borst, 1989). Decreased drug accumulation secondary to increased efflux reduces intracellular concentrations to sublethal levels and confers the multidrug-resistant phenotype (Altenberg et al., 1994; Fojo et al., 1985; Ling & Thompson, 1974). The latter is characterized by resistance to the vinca alkaloids, the anthracyclines, the epipodophyllotoxins, and paclitaxel, which are structurally unrelated hydrophobic natural products (Endicott & Ling, 1989; Van der Bliek & Borst, 1989; Pastan & Gottesman, 1987). Clinical studies are underway testing the therapeutic usefulness of antagonists which are able to block Pgp-mediated efflux (Skelan et al., 1990; List et al., 1993; Miller et al., 1991; Sikic, 1993).

The function of P-glycoprotein in normal cells and the identity of its endogenous substrates remain obscure. Disruption of the mouse homologue of Pgp (*mdr1a*) leads to disruption of the blood brain barrier and increased sensitivity to drugs (Schinkel et al., 1994). The mRNA which encodes Pgp, *mdr1*, is expressed at high levels in several normal human tissues including the adrenal cortex, adrenal medulla, colon, liver, and kidney (Fojo et al., 1987; Arceci et al., 1990). Pgp is present in adult human adrenals but not in fetal and neonatal adrenals and is more strongly expressed in the cortex than in the medulla (Sugawara et al., 1988). Pgp has been postulated to influence adrenal maturation and physiology (Sugawara et al., 1988). Progesterone, corticosterone, and cortisol inhibit [^{3}H]azidopine photoaffinity labeling of Pgp extracted from the endometrium of the gravid mouse uterus and inhibit the specific binding of ^{3}H-progesterone to Pgp (Yang et al., 1989; Qian & Beck, 1990). Progesterone can also inhibit Pgp-mediated vinblastine efflux, although progesterone transport by Pgp has not been demonstrated (Yang et al., 1990).

Numerous studies indicate that phosphorylation of Pgp modulates Pgp-mediated drug efflux. For example, in multidrug-resistant cells, increased protein kinase C (PKC) activity enhances phosphorylation of Pgp and increases Pgp-mediated vinblastine efflux, while decreased PKC activity has the opposite effect (Fine et al., 1988; Center, 1985; Chambers et al., 1990, 1992, 1994; Bates et al., 1993). Short-term TPA or dibutyryl phorbol ester treatment activates PKC, increases Pgp phosphorylation, and reduces the accumulation of [^{3}H]-vinblastine (Chambers et al., 1990; Fine et al., 1988). In contrast, inhibition of Pgp phosphorylation following sodium butyrate treatment increases the accumulation of

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[1] Abbreviations: Pgp, P-glycoprotein; PKC, protein kinase C; MPA, medroxyprogesterone acetate; R_f, retention factor.

vinblastine, adriamycin, and actinomycin D (Bates et al., 1992). Inhibition of PKC with calphostin C, staurosporine, or long-term TPA treatment decreases Pgp phosphorylation and increases the accumulation of vinblastine (Bates et al., 1993). In these studies, however, differential effects were observed. Following sodium butyrate treatment, colchicine transport was preserved, and following calphostin C treatment, verapamil transport was increased. These studies suggested that changes in phosphorylation modulate the transport of different drugs differently.

The present study investigated the interaction of steroids with Pgp in SW620 Ad300 cells, which have been shown to exhibit classic Pgp-mediated multidrug resistance (Lai et al., 1991; Bates et al., 1991). Three aspects of steroid−Pgp interaction were examined: (1) the transport of [^{3}H]steroids by Pgp, (2) antagonism by unlabeled steroids of Pgp-mediated vinblastine efflux, and (3) modulation by Pgp phosphorylation of Pgp−steroid interactions. On the basis of earlier data (Ueda et al., 1992; Yang et al., 1989), we hypothesized (1) that steroid transport by Pgp correlates with steroid hydrophobicity, (2) that steroid antagonism of Pgp-mediated transport correlates with steroid hydrophobicity, and (3) that Pgp phosphorylation modulates Pgp−steroid interactions. In confirming these hypotheses we observed Pgp-mediated transport of 12 [^{3}H]steroids and antagonism by 26 different steroids. Both transport and antagonism correlated with steroid hydrophobicity. Thus, the transport of (and antagonism by) steroid molecules is a general property of Pgp. We also showed for the first time that calphostin C, an inhibitor of Pgp phosphorylation, inhibits photolabeling of Pgp by progesterone, increases [^{3}H]progesterone efflux, and modulates steroid antagonism of Pgp.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Vinblastine (11−21 Ci/mmol), [^{3}H]progesterone (109 Ci/mmol), [^{3}H]-17-hydroxyprogesterone (65 Ci/mmol), [^{3}H]androstenedione (97 Ci/mmol), [^{3}H]cortisol (76 Ci/mmol), [^{3}H]-5α-dihydrotestosterone (116 Ci/mmol), [^{3}H]dexamethasone (89 Ci/mmol), and [^{3}H]azidopine (48 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). [^{3}H]Verapamil (69.3 Ci/mmol), [^{3}H]aldosterone (77.4 Ci/mmol), [^{3}H]pregnenolone (23.5 Ci/mmol), [^{3}H]cholesterol (74.1 Ci/mmol), [^{3}H]dehydroepiandrosterone (88.6 Ci/mmol), [^{3}H]corticosterone (72.5 Ci/mmol), [^{3}H]estradiol (150 Ci/mmol), [^{3}H]testosterone (137 Ci/mmol), [^{3}H]-11-deoxycortisol (48.3 Ci/mmol), and [^{3}H]medroxyprogesterone acetate (MPA) (44.9 Ci/mmol) were obtained from DuPont-NEN (Boston, MA). 16α-Methylprogesterone and Δ^1-progesterone were obtained from Steraloids (Wilton, NH). All other steroids, quinidine, sodium azide, 2-deoxy-D-glucose, and verapamil were obtained from Sigma Chemical Co. (St. Louis, MO), and cyclosporine A was obtained from Sandoz Pharmaceutical. Calphostin C was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). The silica gel 60 F_{254} TLC plates with concentrating zone were purchased from Merck (Darmstady, Germany).

Cell Culture. SW620 human colon carcinoma cells were maintained in RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 2 mM L-glutamine, 10 units of penicillin/mL, 10 μg of streptomycin/mL, and 10% fetal bovine serum (GIBCO). SW620 Ad300 cells were derived from SW620 cells by gradually increasing the adriamycin concentration and were maintained in medium containing 300 ng of adriamycin/mL (Lai et al., 1991). The SW620 Ad300 cells express high levels of P-glycoprotein and possess a multidrug-resistant phenotype characterized by cross-resistance to a wide range of natural products. H295 human adenocarcinoma cells were obtained from Adi Gazdar (University of Texas, Health Sciences Center) (Bates et al., 1991).

Drug Accumulation and Efflux Studies. Cell monolayers were prepared by plating (2−5) × 10^5 cells in 5 mL of medium in the top three wells of a six-well plate 1−2 days before the assay. [^{3}H]Vinblastine or [^{3}H]progesterone accumulation was measured at 37 °C in serum-free medium (Dulbecco's modified Eagle's medium, 1 g of glucose/L, 25 mM HEPES, pH 7.4) as previously described (Fojo et al., 1985). In experiments that examined the effect of calphostin C, cells were incubated for 1 h prior to the accumulation assay at 37 °C under fluorescent light, with or without 500 nM calphostin C. Before starting the assay, cells were washed once with 3 mL of serum-free medium, and then 1.5 mL of serum-free medium with or without 500 nM calphostin C, plus 0.5 μCi of [^{3}H]vinblastine/mL (23−45 nM) or 1 μCi of [^{3}H]progesterone/mL (9 nM) was added to each well. One of the steroids (30 μM), cyclosporine A (3 μg/mL), quinidine (10 μg/mL), or verapamil hydrochloride (10 μg/mL) was added to determine their effect on vinblastine or progesterone accumulation. After a 50 min incubation with [^{3}H]vinblastine or 15 min with [^{3}H]progesterone, the cells were washed twice with cold phosphate-buffered saline, trypsinized, and transferred for scintillation counting. Cell counts were determined for control wells and for wells treated with calphostin C. [^{3}H]Vinblastine and [^{3}H]progesterone efflux studies were performed in the same manner as the accumulation studies, except after the two washings with cold PBS, 1.5 mL of serum-free medium was added and removed at 0.5 min and transferred to a scintillation vial for counting. This last step was repeated at 1 min intervals for 10 min before trypsinizing the cells and counting the remaining tritiated compound.

Drug Uptake. Cell monolayers were prepared by plating (2−5) × 10^5 cells in 5 mL of medium in the top three wells of a six-well plate 1−2 days before the assay. Cells were incubated for 20 min prior to the start of the uptake assay at 37 °C in serum- and glucose-free medium (Dulbecco's modified Eagle's medium, pH 7.4) with or without 15 mM sodium azide and 50 mM 2-deoxyglucose. 1 mL amount of serum- and glucose-free medium, with or without 15 mM sodium azide and 50 mM 2-deoxyglucose, containing either [^{3}H]progesterone or [^{3}H]corticosterone (7−10 nM) was added to each well. After 10, 20, 30, 40, 60, or 120 s, the medium was removed and the cells were washed twice with cold phosphate-buffered saline, trypsinized, and transferred for scintillation counting.

Retention Factor. Thin-layer chromatography of steroids was performed on silica gel 60 F_{254} plates in chloroform:ethyl acetate (2:1) or chloroform:methanol (95:5). Steroid (10 mM) dissolved in methanol (or methanol and water) was applied to plates in separate lanes and run in a vapor-saturated TLC chamber. Steroids were detected using UV light and confirmed by sulfuric acid reaction. Retention factors were determined by the relative mobility of each steroid in the solvent system.

Photoaffinity Labeling. Crude membranes prepared from cells incubated with or without calphostin C at 37 °C for 1 h under fluorescent light were used in the photolabeling

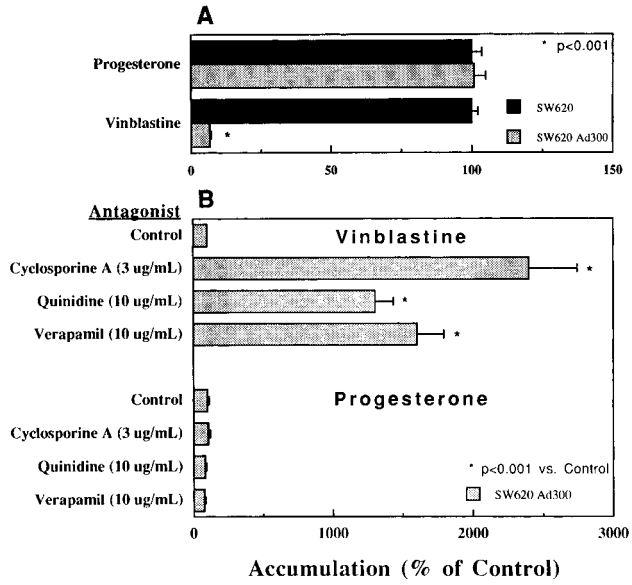

FIGURE 1: Panel A: [^{3}H]progesterone and [^{3}H]vinblastine accumulation in parental SW620 cells and multidrug-resistant SW620 Ad300 cells which express high levels of Pgp. Panel B: effect of three Pgp antagonists on [^{3}H]vinblastine and [^{3}H]progesterone accumulation.

experiments. Cells were harvested in hypotonic buffer (10 mM NaCl, 20 mM sodium phosphate, 1 mM EDTA, 50 μg of aprotinin/mL, 5 μg of leupeptin/mL, pH 7.0) and disrupted using a sonicator. Following removal of cell debris and nuclei by centrifugation at 1000*g* for 5 min at 4 °C, the supernatant was centrifuged at 100 000*g* for 30 min. The crude membrane pellet was resuspended in 10 mM Tris, 250 mM sucrose, pH 7.4, and 200 μg of protein was incubated with [^{3}H]azidopine or [^{3}H]progesterone for 1 h at room temperature. The membranes were then placed on ice under UV light (at 254 and 365 nm wavelength) for 15−30 min, after which time they were incubated in extraction buffer (10 mM Tris, 10 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM DTT, 0.1% Triton) for an additional 15 min. Loading buffer was added, and proteins were separated by one-dimensional 7.5% SDS−PAGE followed by enhancement with Enlightening (NEN) and autoradiography. As a control for the quantity of Pgp present after calphostin C treatment, 2.5 μg of protein from SW620 Ad300 cells was separated by SDS−PAGE, transferred to nitrocellulose, and immunoblotted as previously described using the 4007 antibody (Tanaka et al., 1990).

Statistical Analysis. Steroid accumulations were compared by the two-tailed unpaired Student's *t*-test. Drug efflux and uptake rate studies were evaluated by analysis of variance.

RESULTS

We confirmed previous observations that progesterone is not transported by Pgp (Ueda et al., 1992; Yang et al., 1990). Figure 1A shows drug accumulation studies in parental SW620 human colon carcinoma cells and in a multidrug-resistant subline, SW620 Ad300. Accumulation of [^{3}H]-vinblastine in SW620 Ad300 cells was 6% that of parental SW620 cells, consistent with the high levels of Pgp present in the resistant subline. In contrast, [^{3}H]progesterone accumulation was comparable in the two lines, suggesting that Pgp-mediated progesterone transport was not present. Furthermore, as shown in panel B, the addition of each of three different P-glycoprotein antagonists (cyclosporine A, quinidine, or verapamil) significantly ($p < 0.01$) increased the accumulation of [^{3}H]vinblastine (2398%, 1303%, and 1603%, respectively) in SW620 Ad300 cells but had a negative or no significant effect on [^{3}H]progesterone accumulation (104%, 78%, and 65%, respectively). A similar effect was observed using staurosporine and the cyclosporine analogue, PSC 833 (data not shown).

To examine the effect of Pgp on the accumulation of other steroids, drug accumulation studies were performed using [^{3}H]steroids (12−85 nM) plus [^{3}H]vinblastine and [^{3}H]-verapamil (Figure 2). The accumulation of each compound in the multidrug-resistant SW620 Ad300 subline is expressed as the percent decrease in accumulation relative to that in the parental SW620 cell line. Vinblastine and verapamil accumulations were decreased 93% and 53%, respectively, in drug-resistant SW620 Ad300 cells relative to parental SW620 cells. Progesterone, Δ^4-androstenedione, and medroxyprogesterone acetate (MPA) showed no significant decrease in accumulation. However, all other steroids tested showed a significant ($p < 0.05$) decrease in accumulation in the SW620 Ad300 cells. The glucocorticoids cortisol and dexamethasone showed the greatest decrease in accumulation (46% and 41%, respectively, $p < 0.01$). Similar decreases were displayed by the inactive glucocorticoid precursor 11-deoxycortisol and by the mineralocorticoids corticosterone and aldosterone (36%, 33%, and 44%, respectively, $p < 0.01$). The accumulation of the androgens, testosterone and dihydrotestosterone, was also significantly ($p < 0.05$) decreased. Cholesterol, estradiol, the androgen precursor dehydroepiandrosterone, and the inactive steroids pregnenolone and 17-hydroxyprogesterone were decreased by 14%, 28%, 18%, 14%, and 23%, respectively ($p < 0.01$). These results suggest that Pgp can reduce the accumulation of a wide range of steroids.

To ensure that the differences in accumulation observed were not due to differences in steroid influx in the two cell lines, the uptake of two steroids was determined. Corticosterone and progesterone were chosen as examples of steroids for which Pgp-mediated transport can or cannot be demonstrated (Ueda et al., 1992). The assays were performed in both untreated and ATP-depleted conditions in order to eliminate the impact of Pgp on drug accumulation. Progesterone and corticosterone uptake was determined for up to 2 min after the addition of tritiated compound to untreated and ATP-depleted SW620 and SW620 Ad300 cells (Figure 3). No significant difference in the uptake rate was found between untreated or ATP-depleted cells or between SW620 or SW620 Ad300 cells. Thus, the results of steroid accumulation in SW620 and SW620 Ad300 cells are not affected by the steroid uptake rate.

Compounds can interact with Pgp as substrates and/or as antagonists. The effect of a wide range of steroids as antagonists of vinblastine transport in multidrug-resistant SW620 Ad300 cells was studied. Steroids from the adrenal biosynthetic pathway (upper set, Figure 4) and other steroidogenic compounds chosen for their structural similarities to progesterone (lower set, Figure 4) were evaluated. The accumulation in SW620 Ad300 cells in the absence of any antagonist served as the control, with 100% accumulation. Verapamil, a potent Pgp antagonist at 30 μM, increased [^{3}H]-vinblastine accumulation to 1878% and is shown for comparison. Among steroids, the largest increases were observed with progesterone and progesterone-like compounds. At 30 μM, progesterone increased vinblastine accumulation to 1729%. Progesterone-like compounds,

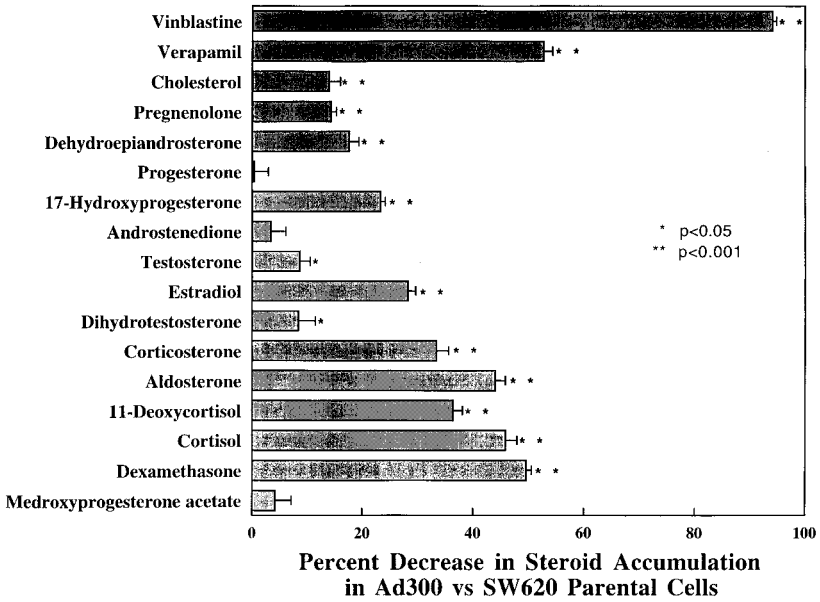

FIGURE 2: Accumulations of [^{3}H]vinblastine, [^{3}H]verapamil, and 15 [^{3}H]steroids are compared. The percent decrease in accumulation in multidrug-resistant SW620 Ad300 cells relative to parental SW620 cells is shown for each compound.

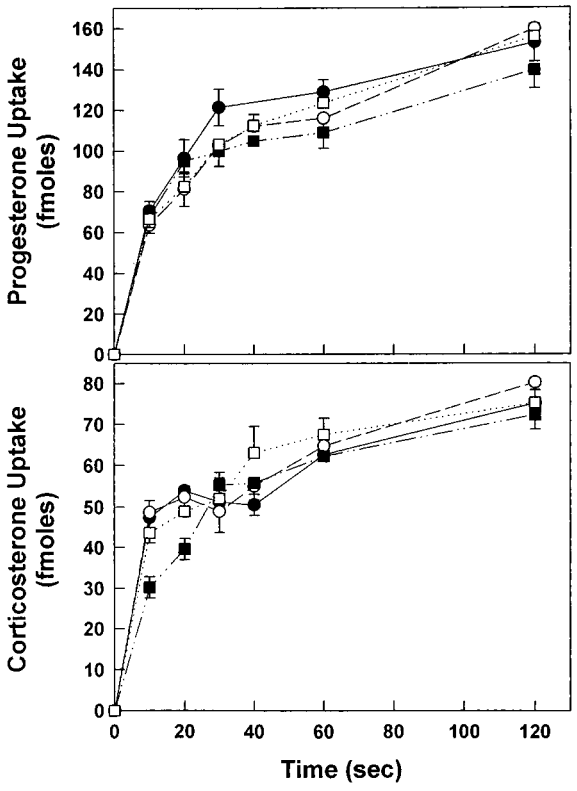

FIGURE 3: Drug uptake in untreated and ATP-depleted SW620 cells (●, untreated; ○, ATP-depleted) and SW620 Ad300 cells (■, untreated, □, ATP-depleted). Both progesterone (upper panel) and corticosterone (lower panel) uptake rates did not differ significantly between the two cell types. Comparable results were observed in both untreated and ATP-depleted conditions.

including medroxyprogesterone acetate, megestrol acetate, 16α-methylprogesterone, Δ^1-progesterone, and 6,16α-dimethylpregnenolone, and the progesterone metabolite, pregnanedione, increased vinblastine accumulation to 2127%, 1321%, 1564%, 1497%, 1872%, and 1587%, respectively. The progestin spironolactone, which is also an aldosterone and androgen antagonist, increased accumulation to 1580%. In contrast, the glucocorticoids (cortisol and 11-deoxycortisol), the mineralocorticoids (aldosterone, corticosterone, and deoxycorticosterone), and the androgens (testosterone, androstenedione, and dehydroepiandrosterone) had smaller effects on vinblastine accumulation (246%, 574%, 150%, 240%, 549%, 264%, 392%, and 203%, respectively) compared to progesterone. Dexamethasone (synthetic analog of cortisol), androsterone (androgen metabolite), dihydrotestosterone (androgen), medroxyprogesterone, and 16-dehydroprogesterone also demonstrated more modest increases in vinblastine accumulation (to 174%, 328%, 260%, 641%, and 552%, respectively).

Having demonstrated reduced accumulation of several steroids in the Pgp-expressing subline and steroid antagonism of Pgp-mediated vinblastine transport, we attempted to correlate these observations with the chemical properties of the steroids. Retention factors (R_f) were calculated in two solvent systems to determine correlations between steroid hydrophilicity or hydrophobicity and Pgp-mediated decreased steroid accumulation or steroid inhibition of vinblastine transport. The R_f of the steroids used in the drug accumulation and vinblastine inhibition experiments were determined in both chloroform:ethyl acetate and chloroform:methanol solvent systems. Figure 5 shows the percent decrease in accumulation for 15 radiolabeled steroids (from Figure 2) versus their R_f values in both solvent systems. The percent decrease in steroid accumulation in multidrug-resistant SW620 Ad300 cells, relative to parental SW620 cells, was inversely proportional to the R_f (hydrophobicity) of the steroid ($r = 0.934$ and 0.962, $p < 0.001$ for both solvent systems). Thus, hydrophilicity enhances Pgp-mediated steroid efflux and decreases steroid accumulation. In contrast, as shown in Figure 6, an increased accumulation of vinblastine, due to inhibition of vinblastine transport (from Figure 4), correlated with increased R_f values and hydrophobicity ($r = 0.677$ and 0.707, $p < 0.001$ for both solvent systems). Together these results suggest that steroid hydrophobicity is most effective for Pgp inhibition, whereas transport requires some hydrophilic properties.

Previous studies have shown that the state of Pgp phosphorylation influences drug transport. Agents that modulate the degree of phosphorylation can influence drug transport. One such agent is calphostin C, a PKC inhibitor, which

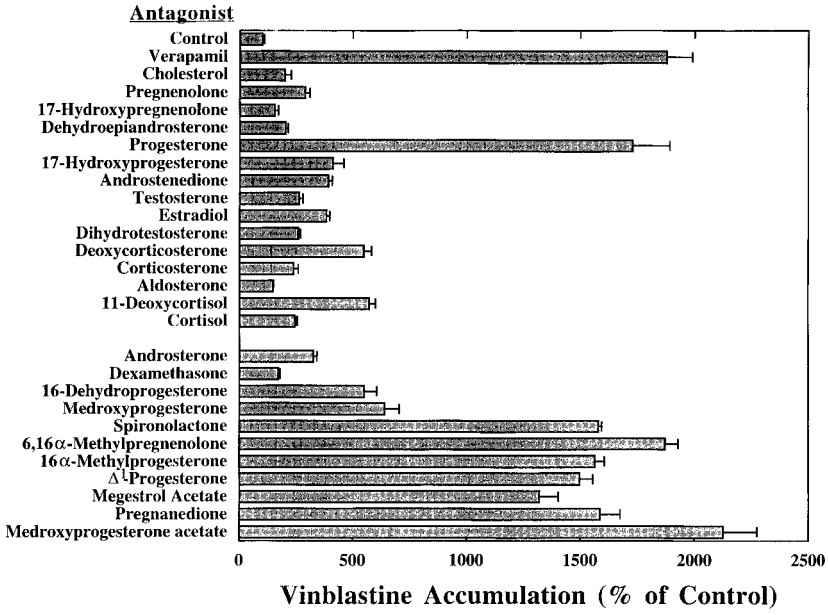

FIGURE 4: Ability of verapamil and 26 steroids to function as Pgp antagonists was determined. The accumulation of [^{3}H]vinblastine in SW620 Ad300 cells, in the absence of an added antagonist (control), in the presence of 30 μM verapamil, or in the presence of 30 μM of each of 26 steroids is shown. The graph depicts the percent increase in vinblastine accumulation relative to the level in control cells, which were arbitrarily assigned a value of 100%.

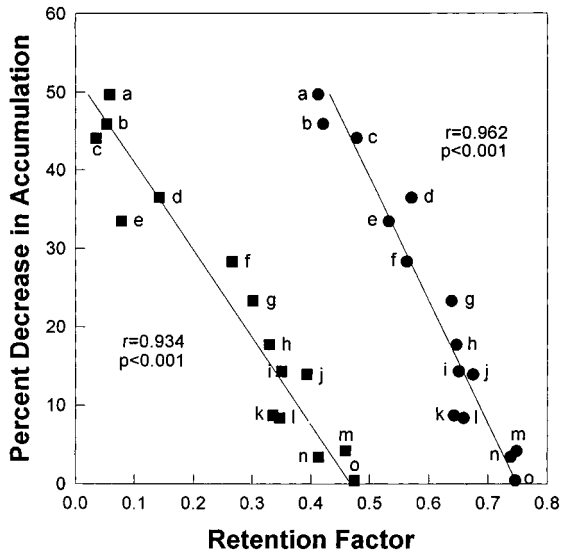

FIGURE 5: Correlations between the percent decrease in steroid accumulation in SW620 Ad300 vs SW620 cells, as shown in Figure 2, and the steroid retention factors (R_f value), obtained using chloroform:ethyl acetate (2:1) (■) and chloroform:methanol (95:5) (●) solvent systems. Higher R_f values indicate greater hydrophobicity in these solvent systems: a, dexamethasone; b, cortisol; c, aldosterone; d, 11-deoxycortisol; e, corticosterone; f, estradiol; g, 17-hydroxyprogesterone; h, dehydroepiandrosterone; i, pregnenolone; j, cholesterol; k, testosterone; l, dihydrotestosterone; m, medroxyprogesterone acetate; n, androstenedione; o, progesterone.

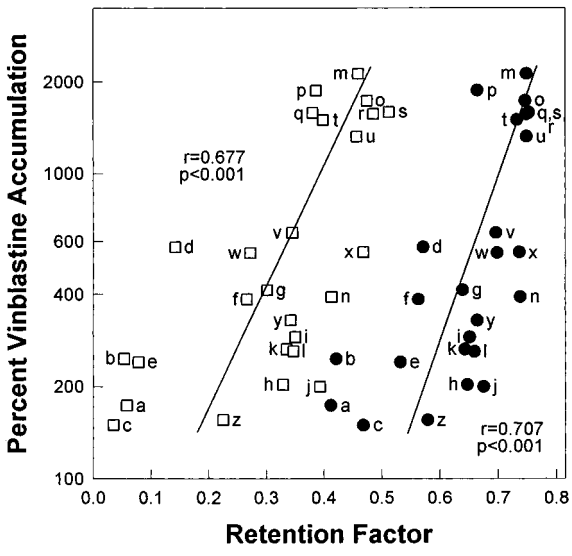

FIGURE 6: Linear regression of the percent of steroid-induced increase in [^{3}H]vinblastine accumulation, as shown in Figure 3, on the steroid retention factors obtained in chloroform:ethyl acetate (2:1) (□) and chloroform:methanol (95:5) (●) solvent systems. Higher R_f values indicate greater hydrophobicity in these solvent systems. Steroids a−o are the same as in Figure 5; p, 6,16α-methylpregnenolone; q, spironolactone; r, 16α-methylprogesterone; s, pregnanedione; t, Δ^1-progesterone; u, megestrol acetate; v, medroxyprogesterone; w, 11-deoxycorticosterone; x, 16-dehydroprogesterone; y, androsterone; z, 17-hydroxyprogesterone.

inhibits Pgp phosphorylation (Bates et al., 1993). Treatment of SW620 Ad300 cells with calphostin C results in increased vinblastine accumulation due to decreased efflux and decreased verapamil accumulation due to increased efflux. To determine the effect of calphostin C on the accumulation of [^{3}H]progesterone, drug accumulations in untreated cells and cells which had been pretreated for 1 h with 500 nM calphostin C were performed. Vinblastine accumulation in SW620 Ad300 cells increased 190% following calphostin C treatment ($p < 0.01$), as previously demonstrated. In contrast, progesterone accumulation decreased 16% ($p < 0.01$), suggesting that inhibition of Pgp phosphorylation could decrease progesterone accumulation by increasing Pgp-mediated efflux. This effect, while small, was highly reproducible in over a dozen experiments. This was confirmed by measuring the rate of progesterone efflux as shown in Figure 7. For these studies, untreated SW620 Ad300 cells and cells pretreated with 500 nM calphostin C were loaded with [^{3}H]progesterone for 15 min and then placed in drug-free medium to determine the rate of efflux. Progesterone efflux occurred more rapidly over the first 5 min in the calphostin C-treated cells ($p < 0.01$).

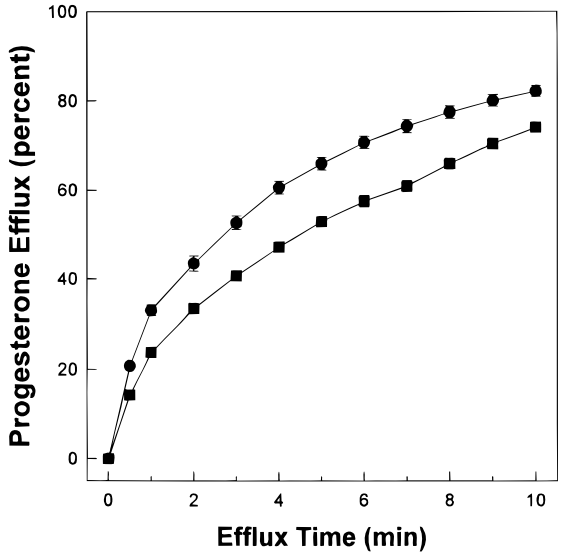

FIGURE 7: [^{3}H]Progesterone efflux from untreated (■) and calphostin C-treated (●) SW620 Ad300 cells. Efflux (as a percent of total initial [^{3}H]progesterone) was measured over 10 min following a 15 min loading period ($p < 0.01$).

To extend these observations, we examined the effect of calphostin C on the inhibition of Pgp-mediated vinblastine transport by steroids. Accumulations of [^{3}H]vinblastine were performed with untreated SW620 Ad300 cells and with cells pretreated for 1 h with 500 nM calphostin C. Figure 8 compares vinblastine accumulation in the presence of 12 different steroids with the accumulation in the presence of verapamil. In untreated cells, as previously shown in Figure 4, most steroids increased [^{3}H]vinblastine accumulation ($p < 0.05$), with the largest increases noted with progesterone and MPA. Treatment with calphostin C to inhibit P-glycoprotein phosphorylation had a complex effect. Alone, calphostin C increased [^{3}H]vinblastine accumulation 349% (relative to control SW620 Ad300 cells). Increased vinblastine accumulation was also observed after calphostin C treatment when the steroids were used as antagonists, with the exception of progesterone and MPA. With progesterone and MPA, as with verapamil, the accumulation of vinblastine in calphostin C-treated cells decreased by 925%, 78%, and 2211%, respectively. These results indicate that calphostin C treatment decreases the effectiveness of progesterone, MPA, and verapamil as Pgp antagonists. Similar studies conducted using unselected H295 adrenocortical carcinoma cells which express Pgp at much lower levels than SW620 Ad300 gave comparable results (data not shown). These assays were conducted in the presence of 500 nM calphostin C following a 1 h pretreatment period. Similar results were observed when the assay was carried out in the absence of calphostin C following the 1 h pretreatment, although the magnitude of the differences was less (data not shown). Previous experiments have also shown that 500 nM calphostin C added to the assay does not affect vinblastine accumulation (Bates et al., 1993).

That calphostin C treatment affects the interaction of drugs with Pgp was demonstrated by photoaffinity labeling studies, as shown in Figure 9. [^{3}H]Azidopine, a radioactive, photoactive dihydropyridine calcium channel blocker, and [^{3}H]-progesterone were used to label Pgp. Previous studies have demonstrated that treatment with calphostin C increases azidopine accumulation (Bates et al., 1993). To allow for precise quantitation, the results were normalized to the level of P-glycoprotein as determined by immunoblotting (Figure 9B). Normalization was used because it was observed that protein extracts isolated from calphostin C-treated cells formed precipitates which impaired migration into the gel. Calphostin C reduced azidopine and progesterone labeling to 19% and 68% of control levels in untreated SW620 Ad300 cells. Similar results were found in H295 cells (data not shown). These results, together with the drug accumulation and efflux studies, suggest that calphostin C treatment can alter drug binding to Pgp.

DISCUSSION

The hypothesis that most steroids are transported by Pgp is supported by the present study, which showed that accumulation of 12 out of 15 tritiated steroids was significantly reduced in SW620 Ad300 cells compared with parental SW620 cells. A decrease in accumulation of greater than 30 percent was observed with cortisol, dexamethasone (a synthetic glucocorticoid), 11-deoxycortisol (a cortisol precursor), and corticosterone and aldosterone (mineralocorticoids). These steroids all have at least two hydroxyl groups and are the most hydrophilic of those tested. Their transport was previously reported to be antagonized by cyclosporin A or verapamil (Ueda et al., 1992; van Kalken et al., 1993; Wolf & Horwitz, 1992). Cholesterol, estradiol, pregnenolone, 17-hydroxyprogesterone, and the androgens (dehydroepiandrosterone, testosterone, and dihydrotestosterone) also showed a significant (between 8% and 30%) decrease in accumulation in SW620 Ad300 cells. In contrast, the accumulation of progesterone, MPA, and Δ^4-androstenedione (an androgen) was not significantly affected by Pgp. When the accumulation of 15 different steroids was examined, a highly significant correlation ($p < 0.001$) between hydrophilicity and decreased accumulation was observed.

The presence of Pgp at high levels in the normal adrenal gland has long produced speculation that it was involved in steroid transport (Fojo et al., 1987; Cordon-Cardo et al., 1990; Thiebaut et al., 1987). However, for many steroids such a role has been difficult to prove. In addition, increased expression of Pgp has been demonstrated in gestational endometrium, suggesting that the interaction of progesterone with Pgp might play a role in pregnancy (Axiotis et al., 1991; Arceci et al., 1988). Multiple investigators have confirmed that progesterone is able to interact with and bind to Pgp while antagonizing the binding and Pgp-mediated transport of other substrates (Arceci et al., 19990; Yang et al., 1989, 1990; Qian & Beck, 1990; Naito et al., 1989; Ichikawa-Haraguchi et al., 1993; Subrahmanyeswara et al., 1994). However, progesterone itself did not appear to be transported (Ueda et al., 1992; Yang et al., 1990). Megestrol acetate was found to be a more potent antagonist than progesterone (Fleming et al., 1992). In contrast, it has been reported that the less hydrophobic steroids cortisol, corticosterone, aldosterone, and dexamethasone are transported by Pgp (Ueda et al., 1992; Wolf & Horwitz, 1992; van Kalken et al., 1993). Our study confirms all of these findings and extends them to a wide range of steroids, demonstrating that more hydrophilic steroids tend to be better transported and to be poorer antagonists while more hydrophobic steroids are more poorly transported and are better antagonists. Further, dephosphorylation of Pgp by calphostin C modulates both the transport and antagonistic potential of the steroids.

Compounds that are poorly transported by Pgp may nevertheless function as Pgp antagonists. Consequently, we

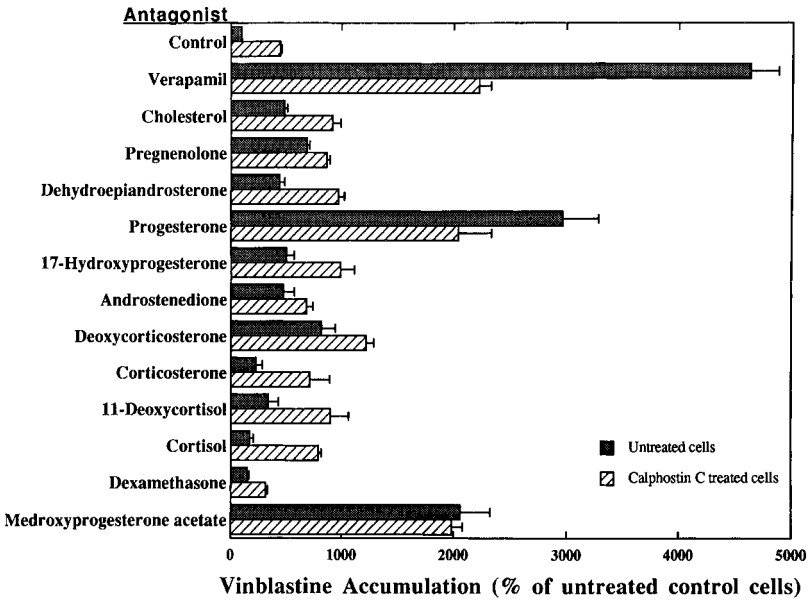

FIGURE 8: [^{3}H]Vinblastine accumulation in untreated and calphostin C-treated SW620 Ad300 cells. The effect of verapamil and 12 steroids on [^{3}H]vinblastine accumulation was evaluated and compared to untreated or calphostin C-treated controls which did not have a potential antagonist present during the accumulation. The level of [^{3}H]vinblastine in untreated controls was arbitrarily assigned a value of 100%.

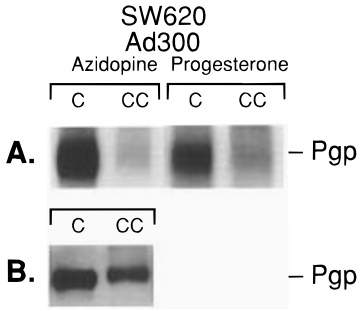

FIGURE 9: Photoaffinity labeling of Pgp from multidrug-resistant SW620 Ad300 cells. (A) [^{3}H]Azidopine and [^{3}H]progesterone were used to label crude membranes isolated from untreated and calphostin C-treated cells. (B) To allow for precise quantitation, the results were normalized to the level of P-glycoprotein as determined by immunoblotting.

evaluated the effectiveness of different steroids as Pgp antagonists. Using multidrug-resistant SW620 Ad300 cells, we measured vinblastine accumulation in the presence and absence of potential steroid antagonists. Progesterone and progesterone-like steroids increased the accumulation of vinblastine to 2100%; no other adrenal or gonadal steroid increased accumulation greater than 575%. In the case of progesterone, antagonism was affected by structural modification of carbon 16 or 17. Medroxyprogesterone, which contains the more hydrophilic 17α-hydroxyl group, increased accumulation only 641%, whereas medroxyprogesterone 17α-acetate, with its more hydrophobic side chain, increased accumulation to 2127%. The progesterone effect on accumulation was diminished by oxidation of the carbon 16−17 bond to yield 16-dehydroprogesterone, which increased vinblastine accumulation to only 552%. The antagonistic potential of progesterone was not lost with addition of a 16α-methyl group to progesterone nor with conversion to Δ^1-progesterone or megestrol acetate. The potency of megestrol acetate as an antagonist was previously reported to exceed that of progesterone in multidrug-resistant KB cells (Fleming et al., 1992). Pregnenolone was a less effective antagonist, increasing vinblastine accumulation to only 289%. However, the addition of more hydrophobic methyl groups at the 6 and 16α positions enhanced the efficacy of pregnenolone and increased vinblastine accumulation to 1872%. Spironolactone, which like progesterone was a good antagonist, has a 7α-acetylthio group and a propionic acid γ lactone that replaces carbon 18−19. As a group, steroids containing hydroxyl groups were poorer antagonists and a steroid's effect on vinblastine accumulation correlated positively with hydrophobicity as determined by thin-layer chromatography. Thus, hydrophilicity decreased steroid accumulation in SW620 Ad300 cells relative to parental SW620 cells, suggesting that hydrophilic residues enhanced Pgp-mediated transport whereas hydrophobicity enhanced a steroid's ability to antagonize Pgp. One possible explanation for these observations is that binding to Pgp is inherent to the steroid structure but that subsequent transport or dissociation depends on its hydrophilicity or hydrophobicity.

Similar findings were reported by Yang and co-workers (1989), who also noted, in an earlier study of five steroids, a correlation between increasing R_f value and inhibition of vinblastine binding to membranes. In addition, structure−function studies related to Pgp antagonism have correlated increasing antagonistic ability with hydrophobicity (Ford et al., 1989; Zamora et al., 1988). The presence of an amino group in a cyclic ring structure, plus one or two aromatic rings, was the typical structure of a Pgp modulator (Ford et al., 1989, 1990; Zamora et al., 1988; Pearce et al., 1989).

Although the present study confirmed previous observations that progesterone blocks but is not normally transported by Pgp, we demonstrated that compounds that modify Pgp phosphorylation can lead to progesterone transport. Previous studies have shown that the phosphorylation state of Pgp can affect drug transport. Calphostin C, a PKC inhibitor, has been shown to decrease Pgp phosphorylation, differentially altering drug transport (Bates et al., 1993). As previously observed, treatment of SW620 Ad300 cells with calphostin C increased vinblastine accumulation and increased verapamil efflux. As in the case of verapamil, increased efflux of progesterone was observed following calphostin C treatment. Thus, dephosphorylation of Pgp

rendered it a poorer vinblastine transporter while enhancing progesterone efflux, albeit to a small extent.

The calphostin C effect on steroid transport was associated with a change in the steroid's efficacy as a Pgp antagonist. As with verapamil, progesterone accumulation decreased after calphostin C and its effectiveness as an antagonist was impaired. In contrast, steroids whose transport decreased after calphostin C had an additive or greater than additive effect on vinblastine accumulation. One possible explanation for these results is that increased drug transport results in less efficient antagonism. However, a straightforward interpretation is difficult because calphostin C treatment is affecting not only the antagonist but also the vinblastine being transported.

We used photoaffinity labeling experiments to examine binding to Pgp (Qian & Beck, 1990). Previous studies have shown progesterone binding to Pgp by photoaffinity labeling, and this was confirmed in SW620 Ad300 cells. Decreased binding of both azidopine and progesterone was observed after calphostin C treatment. However, the loss of binding conferred different outcomes for the two compounds. For progesterone, reduced binding was associated with increased transport, while for azidopine, reduced binding was associated with decreased transport. These differences may be clarified when the role of different phosphorylation sites is understood. Further work is needed to complete our understanding of the physiological and clinical significance of Pgp-mediated transport of steroids and its regulation by phosphorylation.

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