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ANTIESTROGENS AND STEROID HORMONES: SUBSTRATES OF THE HUMAN P-GLYCOPROTEIN

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Abstract—Multidrug-resistant (MDR) tumor cells reduce the toxicity of antineoplastic drugs by an energy-dependent active efflux mechanism mediated by the MDR1 gene product, the P-glycoprotein (Pgp). Pgp expressed in cultured Sf9 insect cells has been shown to exhibit a high capacity ATPase activity in the presence of a variety of drugs known to be transported by the Pgp (Sarkadi *et al.*, *J Biol Chem* **267**: 4854–4858, 1992). The strict dependence of the Pgp ATPase activity on the presence of transport substrates indicates that the drug-stimulated ATPase activity is a direct reflection of the drug transport function of the Pgp. In the present study, this system has been utilized to investigate the possibility that antiestrogens and steroid hormones are transported by the Pgp. Antiestrogens such as tamoxifen, metabolites of tamoxifen (4-hydroxytamoxifen and *N*-desmethyltamoxifen), droloxifen, and toremifene stimulated the Pgp ATPase activity, and the maximum stimulation obtained with these agents equalled the maximal stimulation obtained by the best known MDR chemosensitizer, verapamil. Clomifene, nafoxidine and diethylstilbestrol also stimulated the Pgp ATPase activity, with maximal activations 75, 60 and 45% of the verapamil stimulation, respectively. Different degrees of stimulation of the Pgp ATPase activity were also obtained in the presence of steroid hormones such as progesterone, β-estradiol, hydrocortisone, and corticosterone. Among these, progesterone is a potent inducer of the Pgp ATPase activity; at 50 μM, this hormone stimulated the Pgp ATPase activity as effectively as verapamil. These results suggest that the antiestrogens and steroid hormones that are known to reverse the multidrug-resistant phenotype do so by directly interacting with Pgp, thus interfering with its anticancer drug-extruding activity.

Key words: MDR; P-glycoprotein; drug transport; antiestrogens; steroids; progesterone

Resistance of malignant tumors to multiple chemotherapeutic agents is a major cause of treatment failure. Tumors initially sensitive to cancer drugs often progressively develop resistance to a broad spectrum of structurally unrelated cancer drugs in the phenomenon known as MDR§. The molecular mechanisms underlying drug resistance are diverse. However, the most thoroughly studied drug resistance mechanism is Pgp-mediated drug efflux. The establishment of the Pgp as an energy-dependent drug efflux pump in MDR tumor cells is one of the most important advances in antineoplastic pharmacology during the past decade [1–3]. Moreover, the demonstration by Tsuruo *et al.* that MDR could be reversed by calcium channel blockers such as verapamil [4] raised the possibility that relatively nontoxic drugs for inhibiting the antitumor drug efflux process mediated by the Pgp could be found. Subsequently, other compounds including calmodulin inhibitors [5], immunosuppressants [6], antiestrogens including tamoxifen [7, 8], and steroid hormones [9] were shown to enhance the cytotoxicity of various antitumor agents in MDR cell lines.

Tamoxifen is widely used in the treatment of human breast cancer because of its growth-inhibiting activities on tumor cells expressing estrogen receptors [10]. It has been demonstrated that tamoxifen competes for intracellular estrogen receptors, thus interfering with estrogen-regulated processes related to cell growth [11, 12]. However, antiestrogens have also been found to inhibit the growth of some estrogen receptor-negative breast cancer cells [13], suggesting additional mechanisms of action for these agents. In this regard, tamoxifen has been shown to antagonize the activation of cyclic AMP-phosphodiesterase mediated by calmodulin [14]. It has also been reported that tamoxifen binds to a protein termed the ABS protein, which is present predominantly in the microsomal membranes in almost all tissues [15]. The ABS appears to be a growth-promoting histamine receptor, and interaction of tamoxifen with this receptor is thought to induce the antiproliferative effects [16]. Together, these observations may explain the antiproliferative action of tamoxifen in estrogen receptor-negative MDR cell lines.

In any case, the early benefits of antiestrogen treatment in mammary cancer tend to decrease with time as antiestrogen-resistant subpopulations of cancer cells develop. The reason for this is unknown, but it could be due to changes in the affinity of tamoxifen for ABS. Alternatively, in view of its suspected interaction with the Pgp [17], tamoxifen resistance could be due to its extrusion from the MDR tumor cells by the Pgp.

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§ Abbreviations: MDR, multidrug resistance; Pgp, P-glycoprotein; and ABS, antiestrogen binding site.

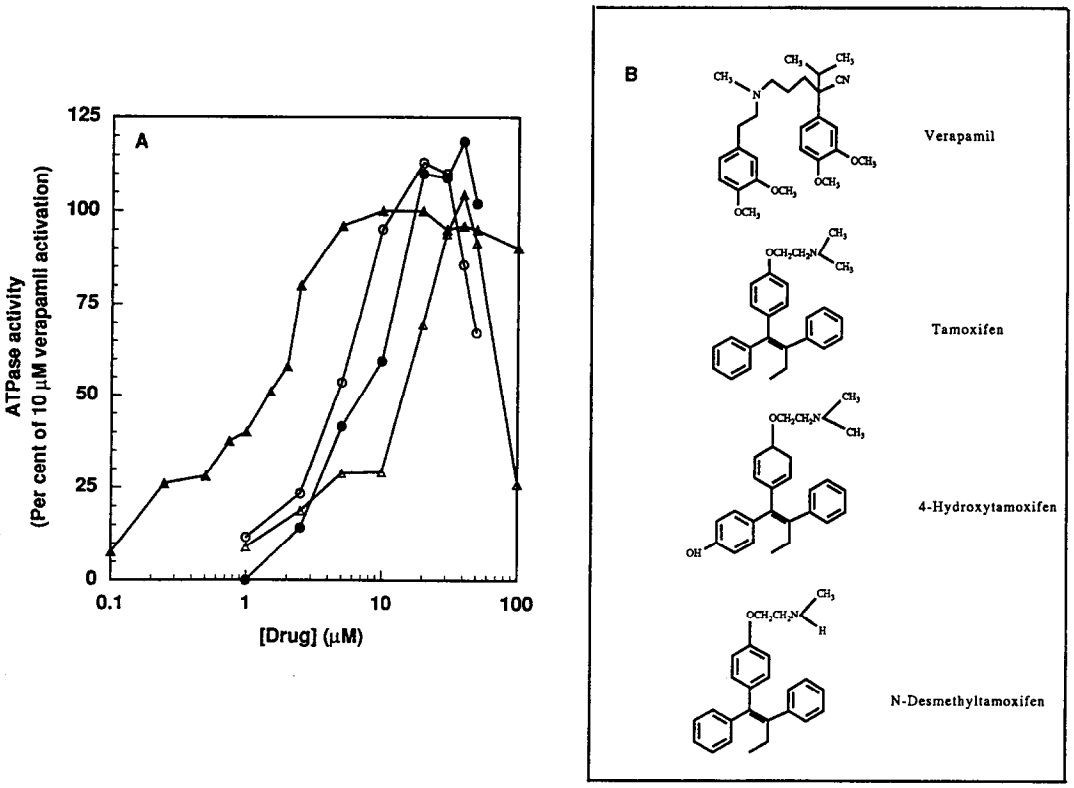

Fig. 1. Effects of tamoxifen and its metabolites on the ATPase activity of the Pgp. Key: (▲) verapamil; (○) tamoxifen; (●) 4-hydroxytamoxifen; and (△) *N*-desmethyltamoxifen. The Pgp ATPase activity measured in the presence of 10 μM verapamil (70 nmol/mg membrane protein/min) was taken as 100%. Activities are expressed as percent of the activity measured in the presence of 10 μM verapamil. Each data point is the average of duplicate determinations.

Drugs capable of interfering with transport catalyzed by the Pgp are potentially valuable clinical agents for reversing MDR and enhancing the effectiveness of many anticancer agents. Traditionally, studies directed toward finding drugs capable of interacting with the Pgp have been identified by screening for compounds able to resensitize drug-resistant cells to the killing action of the previously effective drugs. Though the information obtained from such studies is highly relevant to the overall process of MDR, this approach is heavily biased toward drugs capable of permeating the plasma membrane by virtue of their strongly hydrophobic nature. Thus, there may be many compounds capable of high-affinity interaction with the Pgp that fail as chemosensitizers because they are unable to enter the cells.

Recent work in this laboratory by Sarkadi *et al.* [18] demonstrated that the Pgp expressed in cultured Sf9 insect cells infected with a recombinant baculovirus containing MDR1 cDNA exhibits a high-capacity ATPase activity in the presence of a variety of chemotherapeutic drugs and chemosensitizers. Subsequent studies have demonstrated that the Pgp has essentially no ATPase activity in the absence of transport substrates such as verapamil.* And, importantly, the ability of a variety of compounds to stimulate the Pgp ATPase activity has recently been directly correlated with their ability to be transported by the Pgp [19]. Thus, the drug-dependent Pgp ATPase activity is clearly a direct reflection of the transport function of the Pgp. Because it is a cell-free system, the baculovirus-directed, insect cell Pgp expression system is not subject to the above-mentioned limitations of conventional systems for identifying drugs capable of interacting with the Pgp. Thus, utilizing this experimental system, we are presently attempting to identify nontoxic, high-affinity substrates for the Pgp as potential clinical agents for interfering with its drug extrusion activity. As mentioned above, there are numerous indications that several clinically useful antiestrogens and steroid hormones may be potent Pgp substrates, and in the studies presented herein this possibility is directly explored. The results indicate that various antiestrogens and steroid hormones are indeed effective substrates for the human Pgp.

* Rao US and Scarborough GA, unpublished experiments.

MATERIALS AND METHODS

Materials. Verapamil, clomifene, nafoxidine,

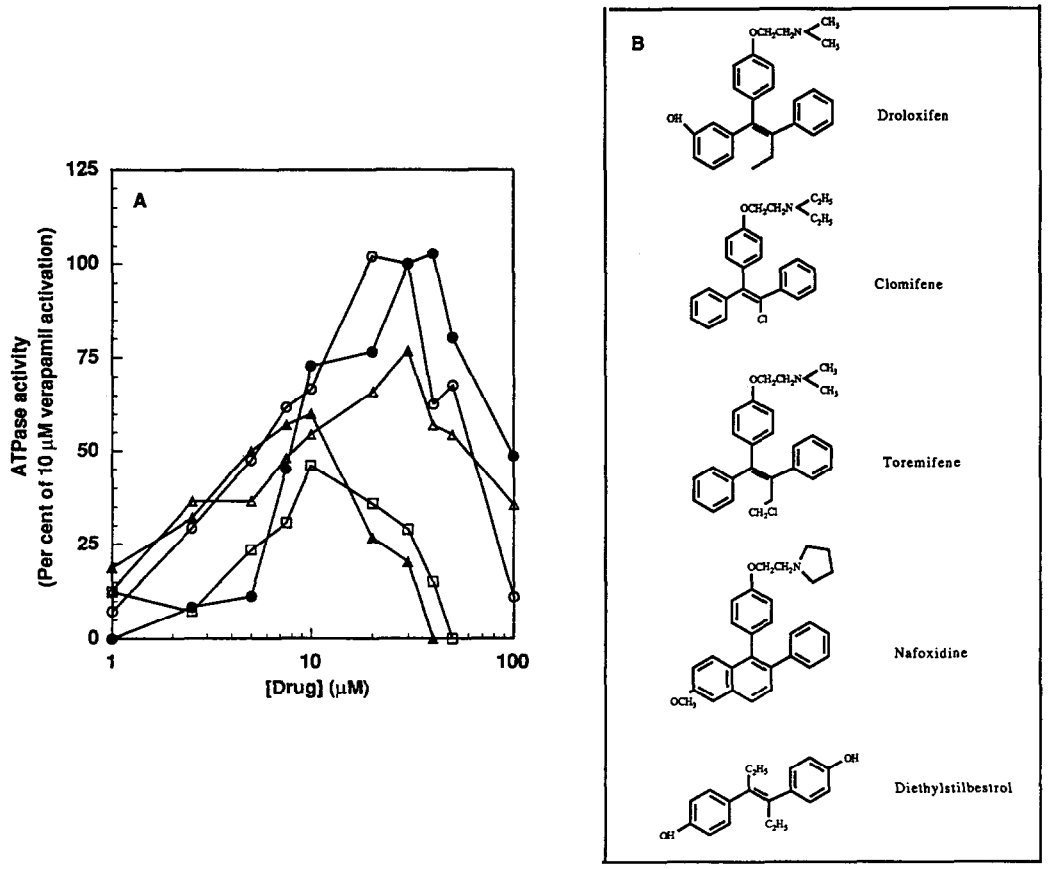

Fig. 2. Effects of several antiestrogens and diethylstilbestrol on the Pgp ATPase activity. Key: (○) droloxifen; (●) toremifene; (△) clomifene; (▲) nafoxidine; and (□) diethylstilbestrol. The Pgp ATPase activity measured in the presence of 10 μM verapamil (70 nmol/mg membrane protein/min) was taken as 100%. Each data point is the average of duplicate determinations.

progesterone, corticosterone, hydrocortisone and β-estradiol were obtained from Sigma. Tamoxifen, 4-hydroxytamoxifen, and *N*-desmethyltamoxifen were from ICI Americas, Delaware. Droloxifen was from Klinge Pharma, GmbH. Toremifene was from Farmos, Finland.

Preparation of Pgp-containing membranes. Sf9 insect cells were infected with the recombinant baculovirus carrying the MDR1 cDNA and grown as described previously [18]. The membrane fraction from the infected Sf9 insect cells was also prepared as described by Sarkadi *et al.* [18] with minor modifications. The cell extract obtained after homogenization and centrifugation at 500 *g* was centrifuged at 15,000 *g* for 15 min at 4°. The pellet was then resuspended in TMEP buffer (50 mM Tris, pH 7.0, with HCl, 50 mM mannitol, 2 mM EGTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/mL chymostatin and 2 mM β-mercaptoethanol) containing 30% glycerol (w/v) and used immediately or saved at −70°. The stored membrane fractions were thawed once and used.

Pgp ATPase activity determination. The Pgp ATPase activity of the membranes was determined in the presence of various drugs as described previously [18]. Membranes prepared from Sf9 insect cells infected with a baculovirus containing *Escherichia coli* β-galactosidase cDNA were used as a control. These membranes do not contain any drug-stimulated ATPase activity. One microliter of the test drug solutions at various concentrations prepared in DMSO was added to 5 μL of membranes (average protein concentration, 2–4 mg/mL) on ice, and the reactions were started by the addition of 95 μL of a reaction mixture to yield final concentrations of 50 mM Tris, pH 6.8, with 2-[*N*-morpholino]ethanesulfonic acid, 2 mM EGTA, 2 mM dithiothreitol, 50 mM KCl, 5 mM sodium azide and 5 mM MgATP. Addition of DMSO alone to the membranes as described above did not affect the drug-stimulated ATPase activity. These experiments were carried out 2–6 times using membranes prepared from different batches of cells with essentially the same results. The standard deviation of each data point calculated from four independent experiments for each compound was in the range of 3–12%.

Protein estimation. Protein was determined by the method of Lowry *et al.* [20] as modified by Bensadoun

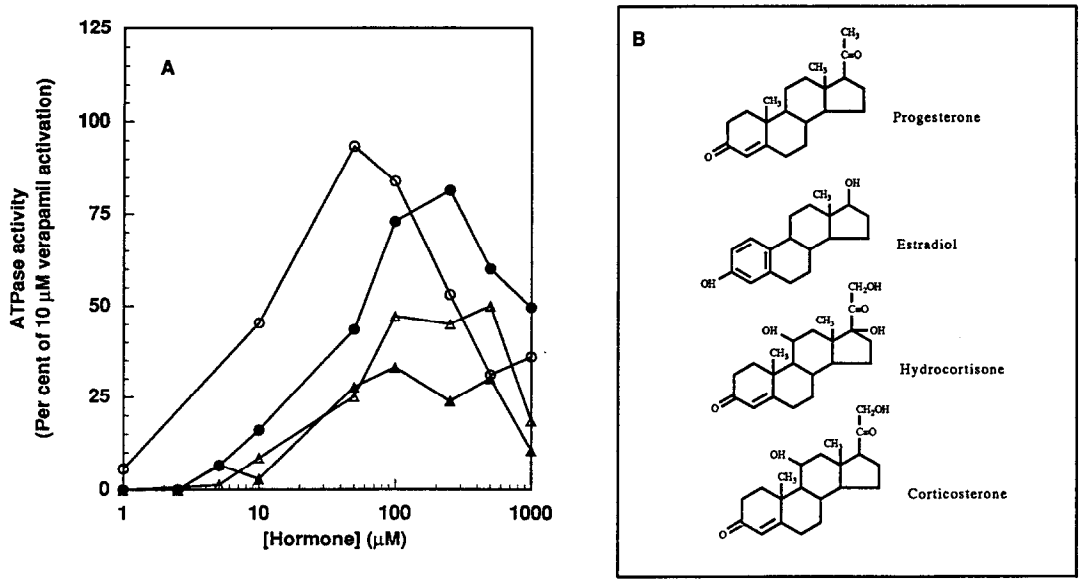

Fig. 3. Effects of steroid hormones on the Pgp ATPase activity. Key: (○) progesterone; (●) β-estradiol; (△) hydrocortisone; and (▲) corticosterone. The Pgp ATPase activity measured in the presence of 10 μM verapamil (70 nmol/mg membrane protein/min) was taken as 100%. Each data point is the average of duplicate determinations.

and Weinstein [21], using bovine serum albumin as the standard.

RESULTS

Figure 1A shows the effect of tamoxifen and its metabolites, 4-hydroxytamoxifen and *N*-desmethyltamoxifen, on the Pgp ATPase activity. The structures of these compounds are shown in Fig. 1B. As a reference, the concentration-dependence curve of verapamil is also shown. In each experiment, maximal Pgp ATPase activity, defined as the activity seen in the presence of 10 μM verapamil, was also measured, and the results were plotted as a percent of this value. Tamoxifen was an effective activator of Pgp ATPase in the 1–20 μM concentration range. The maximum stimulation obtained by 10 μM tamoxifen was comparable to the maximal stimulation of the Pgp ATPase activity by verapamil. The K_a for tamoxifen (concentration needed to bring about half-maximal ATPase activation) calculated from a Lineweaver–Burk plot was approximately 5 μM (not shown). As with most other drugs we have tested [18, *], the Pgp ATPase activity was inhibited at higher concentrations of the drugs. The Pgp ATPase was also stimulated in the presence of the 4-hydroxy derivative of tamoxifen in the range of 2.5 to 40 μM, above which the Pgp ATPase activity was inhibited. Maximum stimulation by 4-hydroxytamoxifen was likewise similar to the maximal stimulation by verapamil, with a K_a of approximately 10 μM. *N*-Desmethyltamoxifen, a metabolite of tamoxifen, also induced the Pgp ATPase activity in the concentration range of 1–40 μM. Pgp ATPase activity at 40 μM *N*-desmethyltamoxifen reached 100% activation.

Figure 2A shows activation of the Pgp ATPase by several other compounds that are structurally related to tamoxifen such as droloxifen (3-hydroxytamoxifen), toremifene, clomifene, nafoxidine and diethylstilbestrol. The structures of these compounds are shown in Fig. 2B. All these compounds were stimulators of the Pgp ATPase; they differed, however, in the extent of their stimulation. Among these, droloxifen and toremifene were potent activators of the Pgp ATPase activity, reaching 100% of the verapamil activation at 20 and 40 μM, respectively. Clomifene also induced the Pgp ATPase activity, but only to 75% of the maximal activation obtained with verapamil. Less potent in this series was nafoxidine which stimulated the Pgp ATPase to 60% of maximal stimulation at 10 μM. Diethylstilbestrol was a relatively weak stimulator of the Pgp ATPase activity. In the presence of 10 μM diethylstilbestrol, the ATPase activity reached only 45% of the maximal stimulation.

Figure 3A shows the effects of several steroid hormones on the Pgp ATPase activity. The structures of the hormones are shown in Fig. 3B. Progesterone activated the Pgp ATPase in a concentration-dependent manner, attaining approximately 90% of the maximal activation at 50 μM. β-Estradiol, hydrocortisone and corticosterone were also activators of the Pgp ATPase activity; maximal activities of 80, 50 and 35% were obtained at 250, 250 and 100 μM, respectively. The K_a for progesterone was 10 μM, and the K_a values for the remaining steroids were approximately 50 μM.

* Rao US and Scarborough GA, unpublished experiments.

DISCUSSION

The results of the present study clearly indicate that various antiestrogens are effective substrates of the Pgp. Tamoxifen was a potent activator of the Pgp. Metabolites of tamoxifen, including 4-hydroxytamoxifen and *N*-desmethyltamoxifen, were almost equally as effective. Several other antiestrogens were equally strong Pgp activators. And none of these agents bears a very strong resemblance to verapamil. Collectively, our results indicate that fairly significant modifications of the basic structures of tamoxifen do not seriously affect interaction with the Pgp. Because the physiological effects of these drugs vary, it may be possible to identify combinations of these and other Pgp substrates that would maximize MDR reversal and minimize unwanted side-effects.

The results presented in Fig. 3 indicate that several steroid hormones are also substrates of the Pgp. Interactions of the Pgp with steroid hormones have been demonstrated before [22–25], and the observation that levels of the Pgp in tissues synthesizing steroid hormones are high has led to the suggestion that the physiological role of the Pgp is in steroid transport [22]. The studies reported here are consistent with this notion. Among the effective steroids, progesterone was about as effective as verapamil, with a K_a of about 10 μM and a similar maximum stimulation. β-Estradiol, corticosterone and hydrocortisone also served as substrates for Pgp, but higher concentrations were required for optimal activation, and the extent of activation was lower. The physiological significance of these observations is unclear. A comparison of the structures of the steroid hormones tested (Fig. 3B) revealed that these compounds differ mainly in their extent of hydroxylation. Progesterone has no hydroxyl groups and is about as effective as any drug we have tested thus far. β-Estradiol, hydrocortisone and corticosterone contain two or more hydroxyl groups and are less effective. These observations suggest that modification of the steroid ring system by hydroxylation may play a significant role in the interaction of steroids with the Pgp. Clearly more extensive molecular modeling and structure/function studies are going to be necessary before an understanding of the structural features required for high-affinity interaction with the Pgp emerges. It should be mentioned in this regard, however, that the necessity of a basic nitrogen atom for productive interaction with the Pgp, suggested by Pearce *et al.* [26], is obviated by the fact that progesterone, which has no such entity, is a very effective Pgp substrate.

Recent reports by Yang *et al.* [9] and Ueda *et al.* [27] suggest that although progesterone binds to the Pgp with high affinity and interferes with the drug efflux catalyzed by the Pgp, it is not transported by the Pgp in the MDR cell lines. In view of the recent results of Homolya *et al.* [19], which clearly correlated the drug-stimulated ATPase activity of the Pgp with drug extrusion, the results presented here indicate that progesterone probably is transported by the Pgp. The inability to observe the transport of progesterone by Pgp in the above-mentioned reports could be due to its high-affinity binding to intracellular receptors or to its metabolism. Alternatively, progesterone interactions with Pgp may represent partial reactions of the transport cycle, in which progesterone binding results only in ATP hydrolysis without transport.

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