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Research Article

RU49953: a non-hormonal steroid derivative that potently inhibits P-glycoprotein and reverts cellular multidrug resistance

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Abstract. Progesterone and the antiprogestin RU38486 have been reported as non-transported modulators of P-glycoprotein-mediated drug efflux. However, their hormonal properties limit their potential for clinical trials. The present work shows that some derivatives from either progesterone/RU38486 or estradiol, displaying differential interaction with hormone receptors, bind to P-glycoprotein and chemosensitize the growth of *MDR*1-transfected cells to vinblastine more strongly than does RU38486. Structure comparison of the compounds indicates that the highly hydrophobic estradiol derivative RU49953, which does not interact with any hormone receptor, inhibits P-glycoprotein-mediated drug efflux very efficiently, as monitored by flow cytometry, and prevents drug site photoaffinity labeling by azidopine. It induces a much higher chemosensitization than the well-known P-glycoprotein modulator verapamil, which is itself more efficient than RU38486. RU49953 therefore constitutes a promising new lead for steroid-type modulators of multidrug resistance.

Key words. P-glycoprotein; multidrug resistance; steroid derivative; RU49953; modulator; drug-binding site; drug transport; hormone receptor.

Human MDR1 and mouse mdr1 P-glycoprotein are often overexpressed in cancer cells displaying a multidrug resistance (MDR) phenotype [1]. Their relative abundance in normal cells of pregnant uterus, adrenal glands and placenta [2–6] suggests a role in the secretion/excretion of and protection against steroid hormones. A number of steroids, including cortisol, dexamethasone, corticosterone and aldosterone, are indeed transported [7–9], whereas progesterone is not, and thus behaves as a modulator of cell MDR by inhibiting P-glycoprotein-mediated anticancer drug efflux leading to intracellular drug accumulation [4, 10–13]. Hydrophobic progesterone derivatives, such as megestrol acetate [14, 15], the antiprogestin/antiglucocorticoid abortive hormone RU38486 (also abbreviated RU486) [16, 17], or C-7-substituted compounds [18], have been found to be more active than progesterone in inhibiting P-glycoprotein activity and promoting intracellular drug accumulation. Structure-activity relationships have shown that the 11-hydroxyl group is essential for steroid transport [9, 19] whereas 17-hydroxyl and 3,20-ketones bring additional positive effects. The transported-steroid site has been located within the transmembrane spans 3 to 5 of the P-glycoprotein N-terminal

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transmembrane domain, where mutations alter recognition of 17-hydroxyl and 20-ketone groups [20]. In addition, direct interaction of non-transported hydrophobic steroid modulators is also detectable with cytosolic nucleotide-binding domains from either P-glycoprotein [21, 22] or the homologous transporter of the *Leishmania tropica* parasite [23]. Although estradiol is not able to inhibit P-glycoprotein activity [4], different hydrophobic analogues such as tamoxifen, toremifene or the pure antiestrogens ICI164,384 and ICI182,780 [24–28] behave as modulators of P-glycoprotein-mediated MDR. However, the modulatory efficiency of all these steroid compounds is generally moderate with various MDR cell lines, being similar to, or lower than, the well-established P-glycoprotein modulator verapamil. In addition, a major problem regarding their potential use in clinical trials is obviously related to their hormonal properties due to their ability to bind to specific receptors, such as glucocorticoid and progesterone receptors for progesterone derivatives, and estrogen receptors for estradiol derivatives.

The aim of the present work was to study a series of new hydrophobic derivatives from either progesterone/RU38486 or estradiol, in order to identify compounds with high modulatory efficiency against P-glycoprotein but low interaction with hormonal receptors. The results show that RU49953, an estradiol derivative with more hydrophobic substitutions than RU38486, chemosensitizes *MDR*1-transfected NIH3T3 cells much more efficiently than verapamil, by interacting at the drug-binding site of the transporter and preventing cellular drug efflux, without any ability to bind to hormone receptors.

Materials and methods

Materials

RU38486 was obtained as previously described [21], and the other steroid derivatives (see fig. 1) were prepared as described elsewhere [29, 30] and supplied by Dr. P. van de Velde (Aventis, Romainville, France). ATP, vinblastine, verapamil and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma. [^{3}H]-azidopine (52 Ci/mmol) was from Amersham. The C219 monoclonal antibody, directed against P-glycoprotein, was from Calbiochem.

Cell cultures

The NIH3T3 drug-sensitive parental cell line is a continuous cell line of contact-inhibited cells derived from NIH Swiss mouse embryo cultures. The NIH-MDR-G185 cell line is an NIH3T3 cell line transfected with human *MDR1* and maintained in the presence of 60 ng/ml of colchicine [31]. Both cell lines were provided by Dr. I. Pastan (National Cancer Institute, National Institutes of Health, Bethesda, Md.) and were grown in a monolayer at 37 °C in a humidified atmosphere of 5% CO_2, in DMEM supplemented with 10% fetal bovine serum (GIBCO), 5 mM glutamine, 50 units/ml of penicillin and 5 µg/ml of streptomycin. The colchicine-selected CH^RB30 line (630-fold resistant) which overexpresses P-glycoprotein [32] and the drug-sensitive Aux B1 parental cell line were provided by Dr. V. Ling (The British Columbia Cancer Research Center, Vancouver, Canada). They were grown at 37 °C in α-MEM medium supplemented with 10% fetal bovine serum, penicillin (1000 U/ml), streptomycin (1 mg/ml) and 2 mM L-glutamine. The CH^RB30 cell line was maintained in 30 µg/ml colchicine.

Inhibition of P-glycoprotein-mediated daunorubicin efflux

Inhibition by steroid derivatives of P-glycoprotein-mediated daunorubicin efflux was measured by flow cytometry. Wild-type or *MDR1*-transfected NIH3T3 cells were seeded in 24-well plates for 48–72 h before the experiment. For assessment of intracellular drug accumulation, cells were first incubated for 30 min at 37 °C with 2 µM daunorubicin in the presence or absence of reversal agents (steroid derivatives or verapamil as the reference), washed in ice-cold phosphate-buffered saline (PBS) and then further incubated for 30 min at 37 °C in the medium with or without the modulators. After washing in PBS, cells were trypsinized, resuspended in 0.2 ml of ice-cold PBS and their intracellular fluorescence was immediately measured in a Beckton Dickinson FacScan. The suspension was gated to eliminate dead cells and debris, and cellular fluorescence was quantified by scanning the emission between 564 and 606 nm (FL-2) with the Cell Quest software application. Samples of 10^4 cells were analyzed. Daunorubicin accumulation in the resistant line was 12% of the wild-type, taken as 100%.

Modulation of sensitivity to vinblastine

The dose-response curves of NIH3T3 cells and the *MDR1*-transfected cell line were determined by plating the cells at a density of 3.5×10^3 cells/well in COSTAR 96-well plates. After overnight incubation, the medium was removed and replaced by 0.2 ml of fresh medium containing increasing concentrations of vinblastine with or without a fixed concentration of the steroid derivatives (added from a 5–10 mM solution in dimethylsulfoxide), always in duplicate. The final dimethylsulfoxide concentration, which did not exceed 0.1%, was not cytotoxic. After 72 h incubation, cell viability was assessed both microscopically and by the MTT assay [33, 34]. Briefly, 10 µl of MTT as a 5 mg/ml solution in PBS was added to each well, and plates were incubated for an additional period of 4 h. Water-insoluble formazan crystals were dissolved by adding 50 µl of 20% SDS and absorbance was read at 540 nm using a microplate reader (Beckman Biomek 2000). Dose-response curves were plotted considering the absorbance of the cells grown in the presence of the steroid

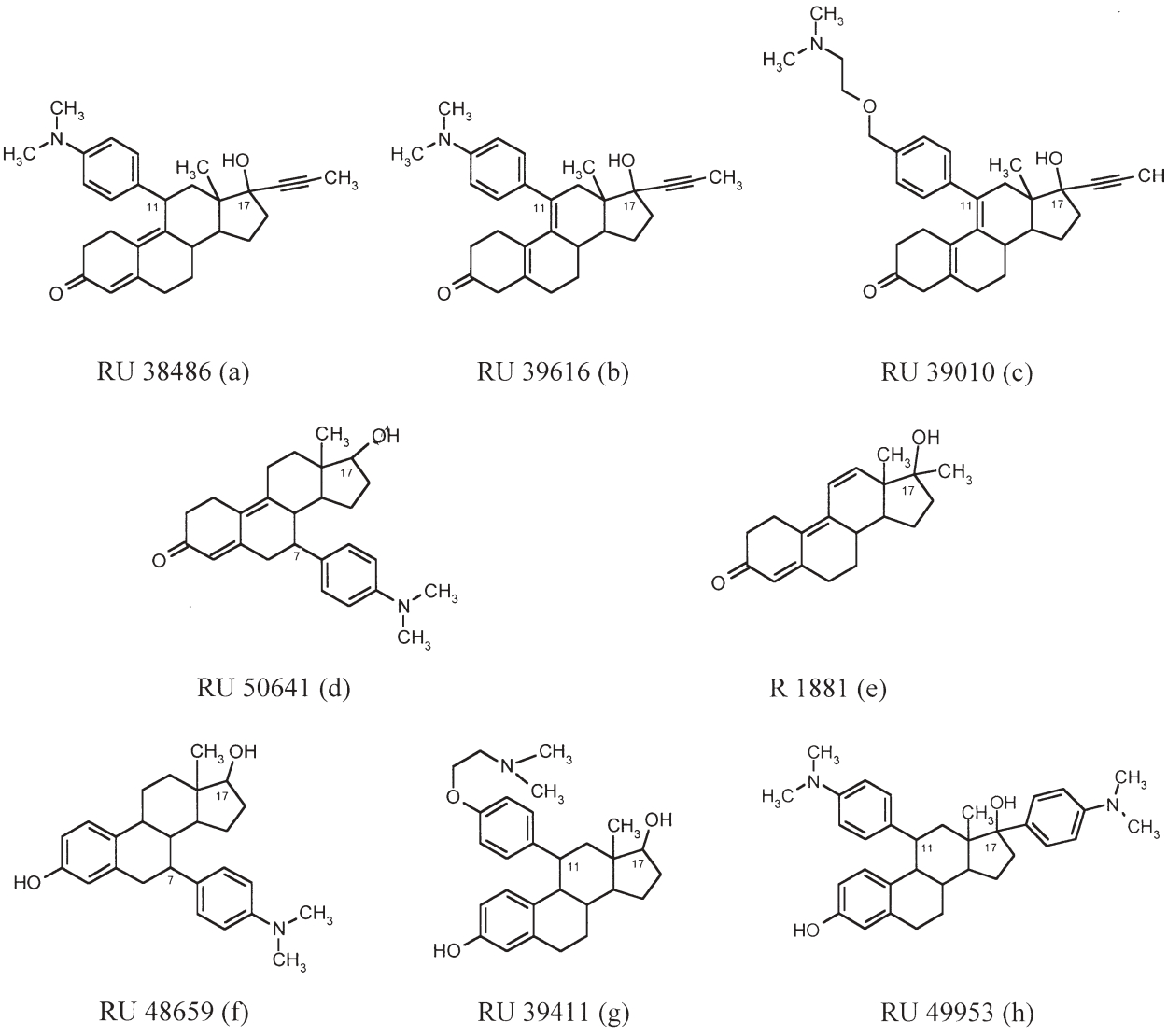

Figure 1. Chemical structures of the different steroid derivatives studied. The upper and middle parts of the figure show analogues of the antiprogestin RU38486 (a–e), whereas the lower part illustrates antiestrogens and derivatives (f–h).

derivative alone as the 100% growth control, thus correcting for intrinsic toxicity. IC_{50} values, defined as the concentrations inhibiting cell growth by 50%, were determined graphically. The resistance index was calculated by dividing the IC_{50} of the resistant line against vinblastine by the IC_{50} of the parental cell line. The reversion index was determined by dividing the IC_{50} of the resistant line against vinblastine alone by the IC_{50} in the presence of vinblastine and 1–10 µM reversal agent (steroid derivative or verapamil).

Inhibition of photolabeling of P-glycoprotein drug-binding sites by azidopine

Parental Aux B1 and resistant CH^RB30 cells were harvested by trypsinization and washed first with α-MEM + 10% fetal bovine serum and then with cold PBS. Cells were suspended in cold PBS containing fresly prepared 2 mM phenylmethanesulfonyl fluoride, and different aliquots containing 10^6 cells were incubated in the dark for 1 h at room temperature with 200 nM [^{3}H]-azidopine (1 µCi) in the presence of 10 or 100-fold molar excess of the steroid derivatives. Samples were exposed to 254 nm UV irradiation for 30 min on ice, and then analyzed by SDS polyacrylamide gel electrophoresis. Each sample was divided into two fractions which were run in parallel and treated separately: one gel was used to measure radioactivity, and the other to perform Western blot and immunodetection with C219 monoclonal antibody.

Determination of characteristics of binding to NBD2

The recombinant C-terminal nucleotide-binding domain (NBD2) from mouse P-glycoprotein was prepared as

published elsewhere [22]. Its direct interaction with steroids was studied by quenching of intrinsic fluorescence as previously described for either flavonoids [22, 35] or protein kinase C effectors [36].

Binding to hormone receptors

The biological samples used to measure steroid-binding affinities for cognate receptor were mouse LMCAT fibroblasts [37] for glucocorticoid receptor (GR), human breast T47-D cells for progesterone receptor (PR), rat prostate for androgen receptor (AR), rat adrenal gland for mineralocorticoid receptor (MR) and human MCF-7 breast cancer cells for estrogen receptor (ER). The cytosoluble extracts were obtained as previously described [38] and the protein concentration was adjusted to 2 mg/ml. Triplicates of tissue/cell cytosoluble extracts prepared in HGDM buffer (10 mM Hepes, pH 7.4, glycerol 10%, 1 mM dithioerythritol, 10 mM sodium molybdate) were incubated for 15 min at 4 °C at a fixed amount of tritiated specific receptor ligand (5 or 10 nM) in the presence of increasing amounts of either ligand or competitor. Radioactive ligands were [^{3}H]-triamcinolone acetonide (38 Ci/mmol; Amersham), specific for the GR, [^{3}H]-R5020 (promegestone, 53 Ci/mmol; Roussel-Uclaf), specific for the PR, [^{3}H]-R1881 (metribolone, 46 Ci/mmol; Roussel-Uclaf), specific for the AR, [^{3}H]-aldosterone (43 Ci/mmol; Amersham), specific for the MR and 17 β [^{3}H]-estradiol (83 Ci/mmol), specific for the ER. The dextran-coated charcoal technique, followed by centrifugation at 8000 g for 5 min, was used to measure the residual binding of tritiated ligand. IC_{50} values were determined from the competition curves.

Results

Inhibition of P-glycoprotein-mediated daunorubicin efflux

The antiprogestin RU38486, whose structure is shown in figure 1, appeared less efficient than verapamil in inhibiting P-glycoprotein drug efflux activity, characterized by intracellular daunorubicin accumulation and monitored by flow cytometry (fig. 2). RU39616 and RU50641 behaved similarly to RU38486, whereas RU39010, R1881 and RU39411 were much less active. RU48659 was more efficient than RU38486, especially at high concentrations (10–30 µM). In contrast, RU39953 was much more potent, even more than verapamil, at all concentrations.

Modulation of sensitivity to vinblastine

MDR1-transfected cells were 30-fold more resistant to vinblastine than the parental cell line in 72-h growth experiments, with respective IC_{50} values of 66.3 ± 23.0 and

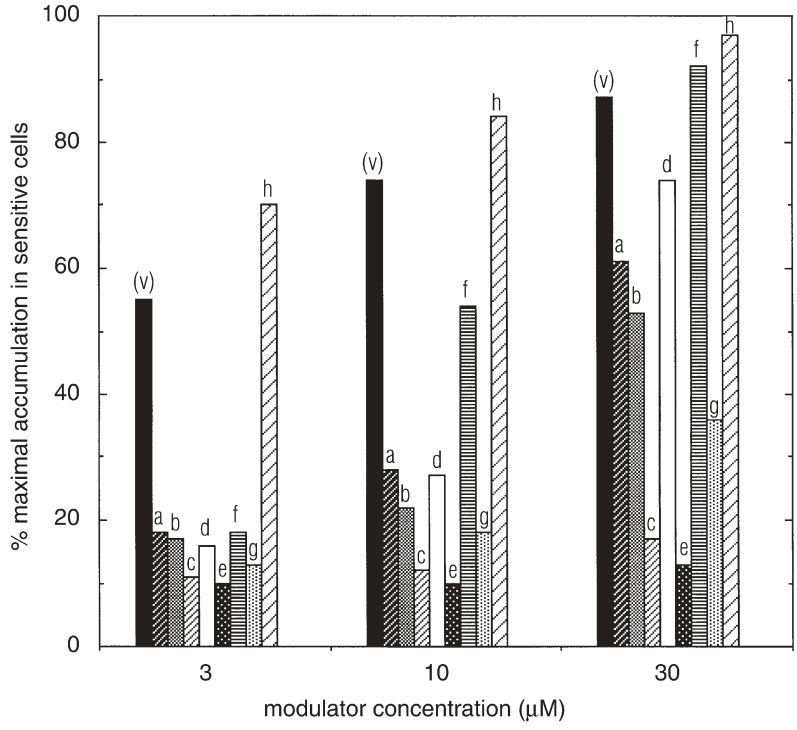

Figure 2. Inhibition of P-glycoprotein-mediated drug efflux leading to intracellular drug accumulation monitored by flow cytometry. *MDR1*-transfected NIH3T3 cells were incubated with daunorubicin either alone or mixed with the indicated modulator compounds: verapamil (v), RU38486 (a), RU39616 (b), RU39010 (c), RU50641 (d), R1881 (e), RU48659 (f), RU39411 (g) or RU49953 (h). After efflux, cells were analyzed by flow cytometry for residual fluorescence.

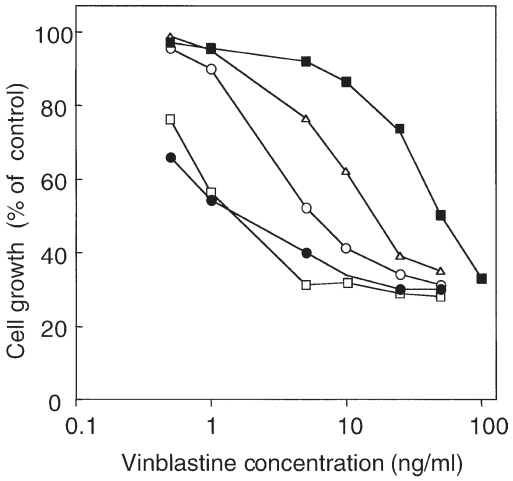

Figure 3. Cell growth chemosensitization to vinblastine in the presence of either RU49953 or RU38486 with reference to verapamil. *MDR1*-transfected NIH3T3 cells were cultured in the presence of the increasing vinblastine concentrations, in the absence of modulator (■) or in the presence of either 3 µM verapamil (○), 10 µM RU38486 (Δ) or 3 µM RU49953 (□). A control NIH3T3 cell growth curve against vinblastine alone is also shown (●).

2.2 ± 0.7 µM (fig. 3). This resistance to vinblastine was abolished by incubation with verapamil, either partially at 3 µM, with a reversion index of 11.2, or completely at 10 µM (table 1). RU49953 was found to be a strong chemosensitizer, completely abolishing the resistance to vinblastine at 3 µM, with a nearly half-maximal effect at 1 µM. In contrast, RU38486 was considerably less potent, even at 10 µM (reversion index of 2.8). When estimating the reversion ability at 3 µM for the two steroids by reference to verapamil, RU49953 appeared 2.5-fold more potent whereas RU38486 was 8-fold less efficient.

A complete comparison of all derivatives, together with intrinsic cytotoxicity, is shown in table 1. The different compounds did not exhibit any cytotoxic effects against either the parental or transfected cell line at 1 µM (data not shown). Interestingly, RU49953 displayed a limited toxicity at 3 µM, i.e. under conditions producing a complete reversion of resistance to vinblastine. RU48659, which was more efficient than RU38486, exhibited almost no cytotoxicity. RU39616 and RU50641 displayed similar reversing activity and similar cytotoxicity as RU38486. The remaining three derivatives, RU39411, RU39010 and R1881, did not exhibit any effect at 3 µM and only produced a limited reversion at 10 µM. The *MDR1*-transfected cell line was found to be slightly more sensitive than the parental line to all the steroid derivatives, except RU39411, indicating the absence of cross-resistance for these hydrophobic compounds.

Inhibition of photolabeling of P-glycoprotein drug-binding sites by azidopine

The ability of steroid derivatives to interfere with drug binding was assayed by prevention of specific photolabeling by radioactive azidopine of the drug-binding sites of membrane-inserted P-glycoprotein [39]. Figure 4 shows that RU49953 was very efficient in preventing labeling, since 13 or 3% residual azidopine binding was observed with 10-fold or 100-fold excess, respectively, of RU49953 (lanes 3 and 4, as compared to lane 2). RU38486 was slightly less efficient, since 18 or 7%, respectively, residual binding was observed (lanes 5 and 6). RU39411 was much less active, with respective residual binding of 48 or 25% (lanes 7 and 8).

Direct interaction of steroids with the C-terminal cytosolic domain of P-glycoprotein

The antiprogestin RU38486 has been previously shown by fluorescence quenching to bind to cytosolic domains of P-glycoprotein, in proximity to the ATP site [21], and

Table 1. Reversion of cellular MDR phenotype, and intrinsic cytotoxicity.

	IC_{50} (µM)[a]			intrinsic cytotoxicity[b]	
Compound	1 µM	3 µM	10 µM	3 µM	10 µM
Verapamil	17 ± 4.0 (5.8)	5.0 ± 1.1 (11.2)	3.3 ± 1.7 (33.6)	4.7 ± 2.6	17.1 ± 6.9
RU38486	> 65 (1.0)	49.5 ± 4.5 (1.3)	28 ± 8.2 (2.8)	4.0 ± 1.0	10.4 ± 5.6
RU39616	n. d.	55.5 ± 2.5 (1.2)	24.4 ± 5.4 (3.2)	5.0 ± 1.0	16.5 ± 0.5
RU39010	n. d.	> 65 (1.0)	43.3 ± 9.5 (1.8)	5.0	4.4 ± 1.7
RU50641	n. d.	29.6 ± 8.1 (1.9)	11.5 ± 5.5 (6.0)	0.0	12.2 ± 6.2
R1881	n. d.	> 65 (1.0)	60 ± 13.2 (1.3)	0.0	11.8 ± 0.8
RU48659	n. d.	18.2 ± 4.1 (3.0)	6.0 ± 2.9 (13.0)	0.0	1.6 ± 0.4
RU39411	n. d.	> 65 (1.0)	37.1 ± 8.2 (2.1)	0.0	37.1 ± 16.1
RU49953	7.2 ± 0.4 (13.7)	1.95 ± 0.55 (28.4)	n. d.	11.9 ± 6.4	n. d.

n.d., not determined.

[a] The IC_{50} values of *MDR1*-transfected drug-resistant cells for vinblastine were determined (from at least two independent experiments in duplicate) in the presence of steroid derivative or verapamil at the indicated concentration, and the reversion index shown in parentheses was calculated.

[b] Percent cytotoxicity was determined on wild-type parental cells.

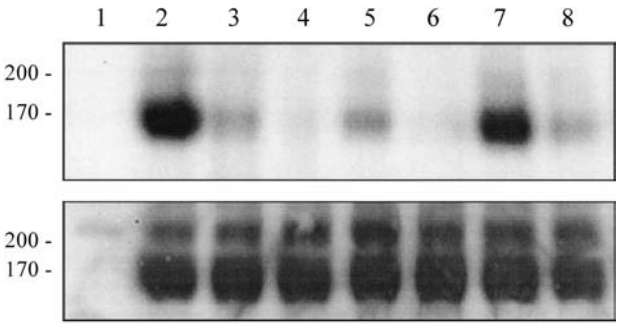

Figure 4. Prevention of azidopine photolabeling. Parental Aux B1 cells (lane 1) or resistant P-glycoprotein-overexpressing CH^RB30 cells (lanes 2–8) were incubated with 200 nM [^{3}H]-azidopine in the absence of modulator (lanes 1 and 2) or the presence of either RU49953 at 10-fold (lane 3) or 100-fold (lane 4) molar excess, RU38486 at 10-fold (lane 5) or 100-fold (lane 6) molar excess, or RU39411 at 10-fold (lane 7) or 100-fold (lane 8) molar excess. Samples were analyzed by SDS polyacrylamide gel electrophoresis, and submitted to either radioactivity counting (upper panel) or Western blot and immunodetection with C219 monoclonal antibody (lower panel). Molecular weights in kDa are indicated on the left.

to partly antagonize the binding of the flavonol kaempferide to C-terminal NBD2 [22]. Figure 5 and table 2 show comparative binding of the different steroid derivatives. RU39616 exhibited a higher binding affinity than RU38486, with an apparent K_D of 4.8 ± 0.66 µM compared to 12.5 ± 1.6 µM, and a similarly high maximal quenching (79.5 ± 4.1% vs 90.1 ± 4.4%). Four other derivatives, RU39010, RU50641, R1881 and RU39411, displayed a much looser binding producing a limited quenching of intrinsic fluorescence, 27–35% at 20 µM by comparison with 57% for RU38486 or 64% for RU39616. Since RU49953 was slightly fluorescent, its binding curve could not be precisely established, but corrections for basal fluorescence led to an approximate binding curve located between those of the two most efficient compounds, RU39616 and RU38486. In contrast, the fluorescence of RU48659 was too high to allow acceptable correction, which prevented any estimation of the apparent binding parameters.

Differential interaction with hormone receptors

Table 2 shows that the antiprogestin RU38486 bound strongly to the PR, GR, AR and MR, while RU50641 bound to the same receptors except for MR. In addition to

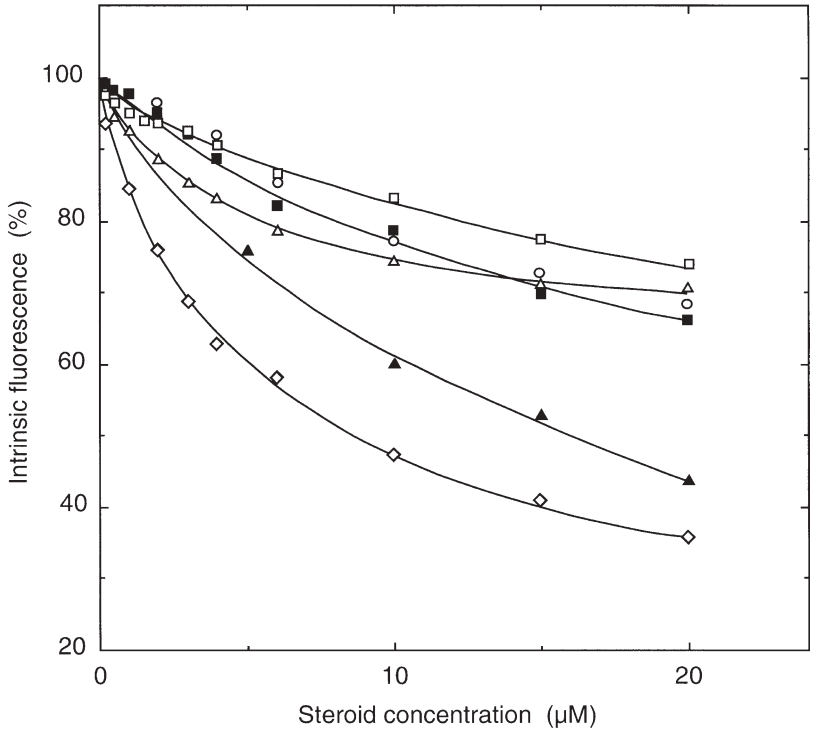

Figure 5. Direct interaction of steroids with P-glycoprotein NBD2. The purified recombinant cytosolic domain, NBD2 [at 1 µM in 20 mM potassium phosphate, 0.5 M NaCl, 20% glycerol, 0.01% 6-O-(N-heptylcarbamoyl)-methyl-α-D-glucopyranoside, 5 mM β-mercaptoethanol, at pH 6.8], was incubated at 25 ± 0.1 °C and mixed with increasing concentrations of either RU38486 (▲), RU39616 (◇), RU39010 (Δ), RU50641 (■), R1881 (○) or RU39411 (□). The excitation was set at 295 nm, and the emission of protein intrinsic fluorescence was recorded from 310 to 360 nm and then integrated. Measurements were corrected for buffer contribution and inner-filter effects of the compounds in parallel experiments with N-acetyltryptophanamide, and the data were analyzed with the Grafit program (Erithacus software).

Table 2. Binding of steroid derivatives to P-glycoprotein NBD2 and to hormonal receptors.

	Binding to P-glycoprotein NBD2[a]		IC_{50} for binding to hormonal receptor (nM)[e]				
Compound	ΔF_{max} (%)	K_D (μM)	GR	PR	AR	MR	ER
RU38486	90.1 ± 4.4	12.5 ± 1.6	2	0.5	4	25	> 1000
RU39616	79.5 ± 4.1	4.8 ± 0.66	1.2	0.4	> 1000	> 1000	500
RU39010	low[b]	–	10	> 1000	100	> 1000	> 1000
RU50641	low[b]	–	12.5	5	12.5	> 1000	> 1000
R1881	low[b]	–	20	> 1000	1.5	> 1000	> 1000
RU48659	n. d.[c]	–	18	> 1000	> 1000	> 1000	25
RU39411	low[b]	–	> 1000	> 1000	> 1000	> 1000	7
RU49953	> 85[d]	6–10[d]	> 1000	> 1000	> 1000	> 1000	> 1000

[a] The binding to purified NBD2 was monitored by quenching of intrinsic fluorescence as described in figure 5. The data were analyzed with the Grafit program to determine the maximal quenching (ΔF_{max}) and the dissociation constant (K_D).
[b] When the ΔF_{max} was low (less than 35% at 20 μM), the K_D was not determined.
[c] RU48659 was highly fluorescent which prevented any accurate determination.
[d] The fluorescence of RU49953 was not too high and could be corrected, giving the approximate indicated parameters.
[e] Receptor binding of steroid derivatives was determined from the curves obtained with increasing amounts of compounds (0.1–1000 nM), for competing the binding of tritiated ligands (5–10 nM) specific for the related receptor. The radioactive ligands were [^{3}H]-triamcinolone acetonide for GR, [^{3}H]-R5020 for PR, [^{3}H]-R1881 for AR, [^{3}H]-aldosterone for MR and 17-β [^{3}H]-estradiol for ER. Each incubation of cytosoluble extracts with radioactive ligands in the presence of increasing amounts of putative competitor was performed in triplicate for 16 h. The bound steroid was separated from the free ligands by dextran-coated charcoal adsorption and centrifugation. Duplicate aliquots of the supernatants were counted for estimating bound ligand. The binding obtained in the absence of competitor was taken as 100%. The IC_{50} was calculated from the competition curves obtained. Given values are the means of six values, and the SES (not mentioned to avoid overloading of the table) were below 10%.

GR, several steroid derivatives bound to either the AR (R1881 and RU39010) or ER (RU48659), whereas RU39411 was specific for the ER. Very interestingly, RU49953 did not bind to any of the hormone receptors studied.

Discussion

This work shows that RU49953, a hydrophobic estradiol derivative with two dimethylaminophenyl substituents at positions 11 and 17, binds strongly to P-glycoprotein drug sites, inhibits its drug-pumping activity and chemosensitizes *MDR1*-transfected cell growth to vinblastine, while not interacting with any hormone receptor and producing a low intrinsic cytotoxicity.

RU49953 was identified from studies conducted with two types of steroid derivatives. First, progesterone derivatives led to rather disappointing results, since the inhibition of P-glycoprotein activity and related modulatory effect were either lower than, or similar to, those produced by RU38486. This indicates the importance of the hydrophobicity brought by the 11-dimethylaminophenyl substituent, which is absent in R1881 or modified in RU39010. In contrast, the positioning of both the dimethylaminophenyl substituent (at position 7, instead of 11, in RU50641) and double bonds (at positions 5 and 9, instead of 4 and 10, in RU39616) appears not to be critical. This is consistent with recent results obtained with hydrophobic substitutions of progesterone at position 7 which decreased P-glycoprotein transport activity leading to intracellular drug accumulation [18]. An increased efficiency was also obtained when the dimethylaminophenyl substituent was introduced at position 21 of either dichlorisone or 17-deoxydexamethasone [40], and with 21-aminosteroid derivatives [41].

Second, estradiol derivatives induced more pronounced effects. When similarly substituted (by 7-dimethylaminophenyl), the estradiol derivative RU48659 was significantly more efficient than the progesterone derivative RU50641 in modulating P-glycoprotein-mediated resistance to vinblastine and inhibiting daunorubicin efflux. Therefore, a phenolic hydroxyl at position 3 appears more efficient than a ketone group, but gives a strong ability to bind to the GR and ER. The increase in MDR modulation related to 7-diethylaminophenyl is consistent with similar effects reported for other hydrophobic substituents at the same position in ICI164,384 [28] and ICI182,780 [24, 26]. Modification of dimethylaminophenyl in RU39411 altered the modulation efficiency with respect to RU48659, in a similar manner to that observed above for the progesterone derivative RU39010 with respect to RU39616. Finally, the presence of two hydrophobic dimethylaminophenyl substituents, at positions 11 and 17 in RU49953, considerably increased the steroid efficiency to inhibit P-glycoprotein drug efflux activity and to revert related cellular multidrug resistance. The effect was much more pronounced than that produced by verapamil taken as a reference. In the case of the *MDR1*-transfected cell line used overexpressing wild-type (G185) P-glycoprotein, verapamil is especially well suited as a modulator against resistance to vinblastine,

and is much more efficient than cyclosporin A [31]. As stressed elsewhere [18], the use of a transfected cell line, in which P-glycoprotein alone is responsible for MDR, in contrast to drug-selected cell lines that may also exhibit increased glutathione transferase and topoisomerase II activities [42, 43], is particularly well suited for studying modulators of P-glycoprotein-mediated MDR. Also worth mentioning is that such hydrophobic substitutions rendered RU49953 completely unable to bind to either the ER or any other hormone receptor. RU49953 was indeed previously found not to bind to the GR [38].

The low ability of RU38486 to revert the MDR phenotype as measured here through a 72-h cell growth chemosensitization to vinblastine, in comparison to the relatively higher effect produced on drug intracellular accumulation as reported previously in the literature and here in figure 2, suggests the presence of secondary targets within the cell and/or low bioavailability at longer time exposures.

The very efficient antagonism of RU49953 against photoaffinity labeling of P-glycoprotein by azidopine suggests that the primary binding site for the estradiol derivative is likely located within the transmembrane domain of the transporter, including membrane spans 3–5 [20]. A lower-affinity interaction was measured with the NBD2 cytosolic domain of P-glycoprotein by comparison to full-length transporter, as previously observed with progesterone hydrophobic derivatives including RU38486 [21, 22]. This cytosolic site might constitute a minor part of the steroid-binding site mainly located within the transmembrane domain. Since RU49953 is much more efficient than RU38486, substituent hydrophobicity appears to be quite important, as discussed above for both progesterone and estradiol derivatives. RU49953 was also reported to inhibit more strongly than RU38486 the transport of the β-amyloid peptide, related to Alzheimer's disease, in P-glycoprotein-overexpressing cells [44], and to inhibit the transport of synthetic glucocorticoids like triamcinolone acetonide and dexamethasone [38].

A good correlation was observed here between the two experimental approaches used to assess steroid derivative effects on drug transport, namely chemosensitization to vinblastine of P-glycoprotein-overexpressing NIH3T3 cells and inhibition of daunorubicin efflux from the same cells. Indeed, the same potency sequence was observed for the different derivatives: RU49953 > RU48659 > RU50641, RU38486, RU39616 > RU39411 > R1881. A comparable efficiency was produced against drug-binding site photolabeling by azidopine for the three derivatives tested: RU49953 > RU38486 > RU39411. The overall results therefore indicate that prevention by steroid derivatives of drug binding to P-glycoprotein is most likely responsible for intracellular drug accumulation and cell growth chemosensitization.

RU49953 exhibited a limited cytotoxicity in wild-type, drug-sensitive NIH3T3 cells, at concentrations producing a marked modulatory effect. For example, a nearly complete reversion was produced by a 3 µM concentration which was 7.3-fold lower than the IC_{50} in the parental line estimated to be 22 µM (not shown here). The fact that the P-glycoprotein-overexpressing cells were not cross-resistant to RU49953 (in fact, these cells were even more sensitive than the parental ones) suggests that the latter compound might not be transported. This is consistent with the lack of transport also reported for RU38486 [16, 19] and tamoxifen [45].

A final interesting feature of RU49953 is its lack of interaction with any hormone receptor in the commonly used concentration range. This contrasts with the strong binding of RU48659, the second most efficient modulator characterized here, to both the GR and ER. Most other steroid derivatives reported as potential MDR modulators also behaved as hormone agonists or antagonists: RU38486 [46], some C-7 progesterone derivatives [47] and 21-dimethylaminophenyl derivatives of deoxydexamethasone and dichlorisone [39] toward the GR and PR; tamoxifen [48], toremifene [49] and the pure antiestrogens ICI164,384 and ICI182,780 [29, 50] toward the ER. In contrast, recently synthesized progesterone derivatives, with quite bulky substituents including two aromatic rings at position 7, were no longer able to bind to the PR [18]. RU49953, an estradiol derivative with two dimethylaminophenyl substituents at positions 11 and 17, was found here not to be able to bind to either the ER or any other hormone receptors, and may therefore constitute a powerful tool for circumventing anticancer drug efflux.

In conclusion, the estradiol hydrophobic derivative RU49953 can strongly inhibit P-glycoprotein drug-pumping activity, and chemosensitize the growth of MDR cells to vinblastine. Such a reversal of MDR is much higher than that produced by the well-known verapamil and all other steroid derivatives reported so far in the literature. RU49953 appears not to be transported by P-glycoprotein, exhibits a limited cytotoxicity in the modulatory concentration range, and does not interact with any hormone receptor. RU49953 can therefore be considered as a new promising lead of steroid-type modulators of P-glycoprotein-dependent MDR in tumor cells.

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