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Rapid report

Potent competitive inhibition of drug binding to the *Saccharomyces cerevisiae* ABC exporter Pdr5p by the hydrophobic estradiol-derivative RU49953

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Abstract

The hydrophobic estradiol-derivative RU49953 inhibits the energy-dependent interaction of yeast multidrug-transporter Pdr5p with its fluorescent drug–substrate rhodamine 6G. The potent inhibition is competitive towards drug binding ($K_i = 23 \pm 6$ nM), whereas nucleoside-triphosphate hydrolysis is two-orders-of-magnitude less sensitive. RU49953 constitutes the most efficient inhibitor of drug binding to a yeast multidrug ABC exporter reported so far.

Keywords: RU49953; Steroid; Pdr5p; *Saccharomyces cerevisiae*; Drug transport; ATPase

1. Introduction

The major multidrug exporter of the yeast *Saccharomyces cerevisiae* is the full-size ABC (ATP-binding cassette) transporter Pdr5p [1]. Its topology is different from that of the mammalian P-glycoprotein in that each of the two cytosolic nucleotide-binding domains is located at the N-terminal side relative to each transmembrane domain [2,3]. Moreover, several important amino acid substitutions are observed in the ATP-binding motifs [2,4]. Nevertheless, Pdr5p interacts with a number of P-glycoprotein substrates [5,6] and modulators such as flavonoids [7] and protein kinase C effectors [8].

Pdr5p-enriched plasma membranes exhibit high ATPase and UTPase activities [9]. In such membranes, the MgATP-dependent binding of rhodamine 6G to Pdr5p can be conveniently monitored by quenching of the probe fluorescence. Interaction of multiple drugs, characterized by alteration of rhodamine fluorescence quenching, correlates to the drug-resistance properties of Pdr5p-overexpressing yeast cells [5].

Steroids are transported in vivo by Pdr5p and homologous transporters in *S. cerevisiae* [5,6,10,11] and *Candida albicans* [12]. In isolated plasma membranes, micromolar concentrations of progesterone and derivatives, as well as of estradiol and deoxycorticosterone [5,7], inhibit the ATP-dependent drug-pumping activity of Pdr5p. Mammalian P-glycoprotein is also known to transport a number of hydroxylated steroids [13–15], whereas more hydrophobic compounds, such as progesterone [16,17], its RU38486 derivative [18,19] and the RU49953 estradiol derivative [20], are not transported by P-glycoprotein but inhibit its drug-pumping activity.

The present results show that RU49953 constitutes the highest, competitive, inhibitor of Pdr5p interaction with rhodamine 6G ever reported. In addition, it produces a

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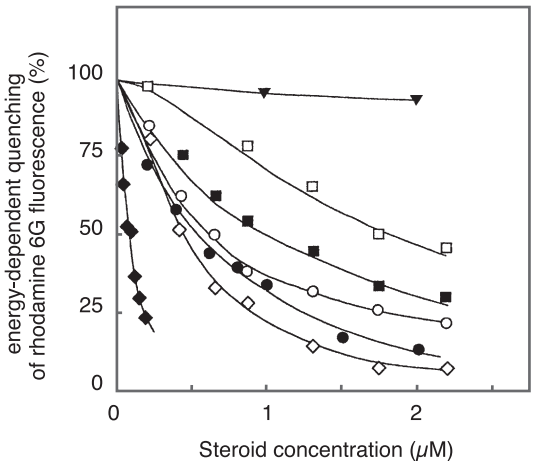

Fig. 1. Inhibition of the energy-dependent interaction of rhodamine 6G with Pdr5p. Pdr5p-enriched plasma membranes were incubated with 144 nM rhodamine 6G and increasing concentrations of RU49953 (♦) or RU38486 (●), or other derivatives such as RU39616 (◇), RU48659 (○), RU50641 (■), RU39411 (□) or R1881 (▼). The reaction was initiated by the addition of 4.8 mM $MgCl_2$ and 4.8 mM ATP, and followed by monitoring the decrease in fluorescence. The IC_{50} values of rhodamine 6G quenching were determined using the Grafit program (Erithacus software).

low, noncompetitive, inhibition of nucleoside-triphosphate hydrolysis.

2. Materials and methods

Steroid derivatives were obtained as previously [20,21]. ATP and UTP were purchased from Sigma, and rhodamine 6G from Merck. Pdr5p-enriched plasma membranes were prepared from the yeast *S. cerevisiae* mutant AD124567 [22] as already reported [7,8].

Energy-dependent quenching of rhodamine 6G fluorescence and nucleoside-triphosphate hydrolytic activity within Pdr5p-enriched yeast plasma membranes were assayed as previously [5,7]. Briefly, Pdr5p-enriched plasma membranes (70–130 μg of protein) were incubated at 30 °C in 2 ml of 50 mM Hepes, pH 7.0, in the presence of rhodamine 6G. The reaction was initiated by 4.4 mM ATP. The decrease in fluorescence at 552 nm was recorded for 20–40 min after steroid addition, and the linear initial slope was estimated. ATPase and UTPase activities were measured by incubating Pdr5p-enriched membranes (10–20 μg of protein) at 35 °C in 500 μl of 50 mM MES, pH 7.5, containing 0.3 mM ammonium heptamolybdate, 75 mM potassium nitrate and 7.5 mM sodium azide, in the presence of 6 mM of both MgCl2 and either ATP or UTP. After 5–15 min, the reaction was stopped by trichloroacetic acid, and the released inorganic phosphate was titrated. Steroid derivatives were added from stock solutions in dimethylsulfoxide, up to a 2–4% (v/v) final concentration.

3. Results and discussion

Fig. 1 shows that the hydrophobic estradiol-derivative RU49953, whose structure is shown in Fig. 2, strongly inhibited the energy-dependent rhodamine 6G interaction with Pdr5p. The IC_{50} (inhibitor concentration producing 50% inhibition) was 0.083 ± 0.007 μM for RU49953, whereas a lower-affinity inhibition was observed for RU38486 ($IC_{50} = 0.54 \pm 0.03$ μM). All the other tested derivatives were less efficient than RU38486, with the exception of RU39616 (an RU38486 analogue with double bonds at positions 5 and 9, instead of 4 and 10), which behaved similarly ($IC_{50} = 0.45 \pm 0.03$ μM). Those compounds included RU48659, an estradiol derivative with a dimethylaminophenyl at position 7 ($IC_{50} = 0.70 \pm 0.05$ μM), RU50641, an RU38486 derivative with a dimethylaminophenyl at position 7, instead of 11, and without substitution at position 17 ($IC_{50} = 1.06 \pm 0.11$ μM), and RU39411, an estradiol derivative with a modified dimethylaminophenyl at position 11 ($IC_{50} = 1.9 \pm 0.2$ μM). The less efficient compound, R1881 (an 38486 derivative without substitution at position 11), hardly inhibited at 2 μM (< 10%) as its IC_{50} value was higher than 25 μM.

As similar structure–activity relationships were obtained previously on P-glycoprotein overexpressing NIH3T3 cells towards growth chemosensitization to vinblastine [20], it appears that mammalian P-glycoprotein and yeast Pdr5p obey similar mechanisms for interaction with drugs even though their different topology has justified their classification in the phylogenetic families B and G, respectively, of

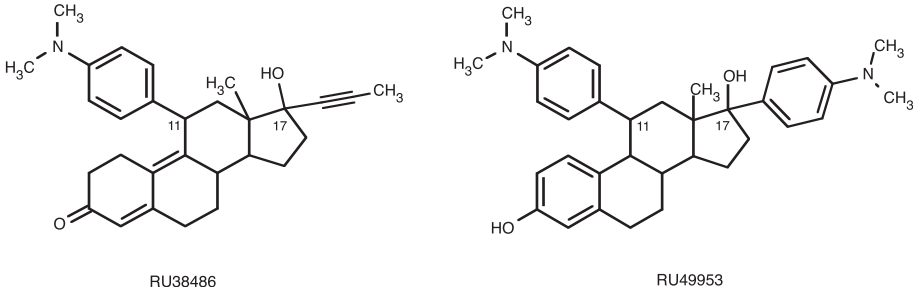

Fig. 2. Chemical structures of the progesterone derivative RU38486 and the estradiol derivative RU49953. RU38486 is a 11-dimethylaminophenyl, 17-propynyl derivative of progesterone whereas the even more hydrophobic RU49953 is a 11,17-di(dimethylaminophenyl) derivative of estradiol.

the ABC-transporter superfamily. This is consistent with the observation that Pdr5p is sensitive to many substrates and modulators, including steroids, known to interact with P-glycoprotein [5,6]. When concentrations of both rhodamine 6G and RU49953 were varied in the nanomolar range (Fig. 3), the competitive inhibition produced by the estradiol analogue allowed to determine a very low K_i value from Dixon plots (23 ± 6 nM) indicative of an extremely high-affinity interaction at the rhodamine 6G-binding sites. To our knowledge, this constitutes the highest affinity ever reported for a Pdr5p inhibitor. Indeed, natural steroids were found to be two-orders-of-magnitude less efficient [5], and lower-affinity inhibitions were also observed with either flavonoids [7], protein kinase C effectors [8] or a variety of other compounds [6].

In contrast, much higher concentrations of steroids were required to inhibit UTPase activity (Fig. 4): an IC_{50} value of 3.6 ± 0.24 μM was obtained for RU49953, while the progesterone derivative, RU38486, was less efficient (IC_{50} = 7.5 ± 0.67 μM). Curiously, the ATPase activity of Pdr5p was even less sensitive to steroid inhibition than UTPase (IC_{50} = 10.0 ± 1.5 μM). A similar differential sensitivity to inhibition between UTPase and ATPase activities was already observed with prenylflavonoids [7,23] and protein kinase C effectors [8], and UTP was much less efficient than ATP to promote rhodamine 6G transport [5]. Inhibition of nucleoside-triphosphate hydrolysis by steroids was further analyzed by varying the concentrations of both UTP and RU49953 (inset, Fig. 4), and found to be noncompetitive.

The fact that RU49953 inhibited 400-fold better drug transport than ATP-driven energy production indicates that RU49953 interacts with high affinity at the drug-binding sites within the transmembrane domain of Pdr5p. Such a binding might have been expected to stimulate nucleoside-triphosphate hydrolysis as observed for P-glycoprotein [24].

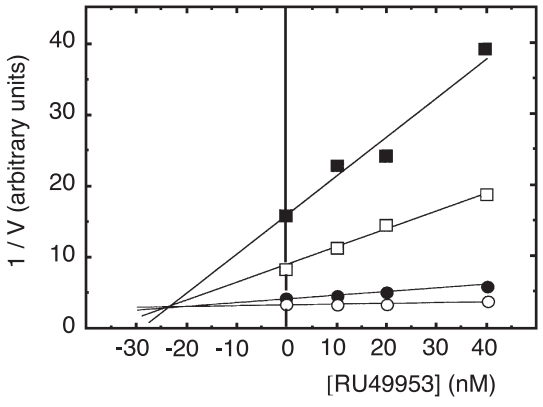

Fig. 3. Type of inhibition by RU49953 of the energy-dependent interaction of rhodamine 6G with Pdr5p. Membranes were incubated, under the same conditions as in Fig. 1, with the indicated RU49953 concentrations in the presence of rhodamine 6G at either 10 (■), 25 (□), 50 (●) or 100 (○) nM, before $MgCl_2$ and ATP addition.

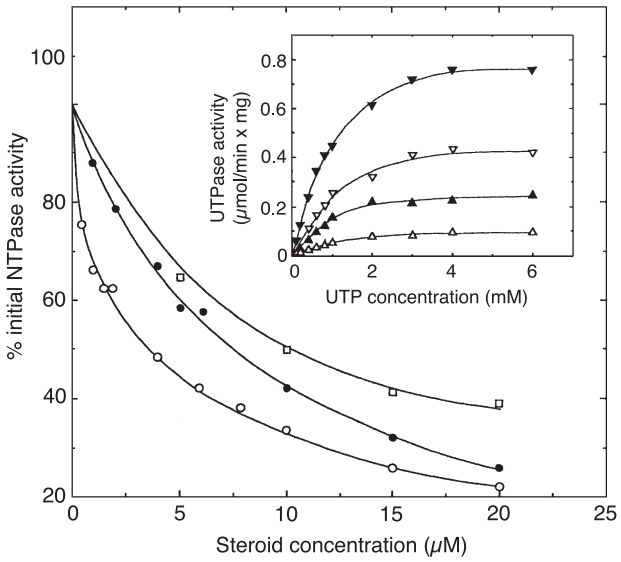

Fig. 4. Inhibition of Pdr5p nucleoside-triphosphate hydrolytic activity. The Pdr5p-enriched plasma membranes were incubated in the presence of either RU49953 (○,□) or RU38486 (●), and the reaction was started by the addition of 6 mM of either UTP (○,●) or ATP (□), and excess $MgCl_2$ providing 1 mM free Mg^{2+}; released phosphate was titrated colorimetrically. Inset: type of RU49953 inhibition on UTPase activity; membranes were incubated with the estradiol derivative at either 0 (▼), 2 (▽), 5 (▲) or 10 (△) μM, and assayed with increasing UTP concentrations as indicated.

This was not the case here for Pdr5p with RU49953, as previously with flavonoids [7,23] and protein kinase C derivatives [8]. This Pdr5p behavior might be related to the probable lack of two fully functional hydrolytic sites [2]. Indeed, in bacterial ABC transporters, the Walker motifs A and B of the N-terminal nucleotide-binding domain together with the motif C/ABC signature of the C-terminal domain bind the same ATP molecule, as demonstrated in the crystal structure of MJ0796 [25]. The putative hydrolytic deficiency in one ATP-binding site of Pdr5p and of all full-size members of the G family (so far restricted to the fungi and plant kingdoms) of the ABC transporters superfamily may modify the coupling mechanism between ATP hydrolysis and drug binding and transport.

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