Prenyl-Flavonoids as Potent Inhibitors of the Pdr5p Multidrug ABC Transporter from *Saccharomyces cerevisiae*†

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ABSTRACT: The Pdr5p multidrug ABC ("ATP-binding cassette") transporter was highly overexpressed in plasma membranes from a yeast strain exhibiting both *pdr1-3* gain-of-function mutation in the transcription factor-encoding gene *PDR1* and disruption of genes encoding other plasma membrane ABC transporters. Solubilized and purified Pdr5p displayed a tryptophan-characteristic intrinsic fluorescence, whose quenching was used to monitor interactions with substrates and effectors. The transporter exhibited a magnesium-dependent binding affinity for ATP and its fluorescent analogue 2′(3′)-*N*-methylanthraniloyl-ATP, producing a marked fluorescence resonance-energy transfer. It also bound a series of known drug substrates and modulators. Interestingly, yeast Pdr5p interacted with flavonoids recently found to bind to cancer cell P-glycoprotein and to the protozoan parasite multidrug transporter. The extent of high-affinity binding of prenyl-flavonoids to purified Pdr5p was correlated to their efficiency to inhibit energy-dependent quenching of rhodamine 6G fluorescence catalyzed by Pdr5p-enriched plasma membranes. The hydrophobic flavonoid derivative 6-(3,3-dimethylallyl)galangin was the most efficient, with a K_i of 0.18 μM for competitive inhibition of the MgATP-dependent quenching of rhodamine 6G fluorescence. In contrast, inhibition of either ATP or UTP hydrolysis occurred at much higher concentrations and appeared to be noncompetitive. Prenyl-flavonoids therefore behave as potent inhibitors of drug binding to the yeast Pdr5p ABC transporter.

Flavonoids inhibit a number of ATP-binding proteins (*1*−*9*), and indeed bind to the ATP site of kinases CDK2 (*10*) and Hck (*11*). Flavonoids exhibit contradictory effects as potential modulators of cancer cell multidrug resistance (MDR);[1] they either decrease (*12*) or increase (*13*) intracellular accumulation of cytotoxic drugs, possibly by acting on different cellular targets. The use of purified recombinant nucleotide-binding domains from mouse P-glycoprotein showed that flavonoids bind not only to the ATP-binding site but also to a region interacting with hydrophobic modulatory steroids (*14*). A high-affinity prenyl-flavone, 8-(1,1-dimethylallyl)kaempferide, was able to inhibit daunomycin efflux from, and to chemosensitize the growth of, multidrug resistant cells of the protozoan parasite *Leishmania tropica* (*15*). With purified and reconstituted P-glycoprotein, it was reported that the inhibition of Hoechst 33342 efflux by the flavone quercetin was, at least partly, due to inhibition of ATPase activity (*16*). The authors recognized, however, that they had no direct way to measure the extent of binding of the flavonoid to the transporter.

In contrast, the yeast Pdr5p multidrug transporter can be highly overexpressed in strains with mutated Pdr1p and Pdr3p transcription factors (*17*, *18*), where other ABC transporters have been eliminated (*19*, *20*). High amounts of purified transporter can be prepared upon detergent solubilization and centrifugation fractionation (*19*, *21*). Such material, which contains a number of tryptophan residues (*22*), is well suited to monitoring direct interactions of Pdr5p with substrates and effectors by monitoring changes in intrinsic fluorescence and fluorescence resonance-energy

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[1] Abbreviations: ABC, ATP-binding cassette; DMA, dimethylallyl; EDTA, ethylenediaminetetraacetic acid; MANT-ATP, 2′(3′)-*N*-methylanthraniloyl-ATP; MDR, multidrug resistance; NBD1, N-terminal nucleotide-binding domain; NBD2, C-terminal nucleotide-binding domain; PDR, pleiotropic multidrug resistance.

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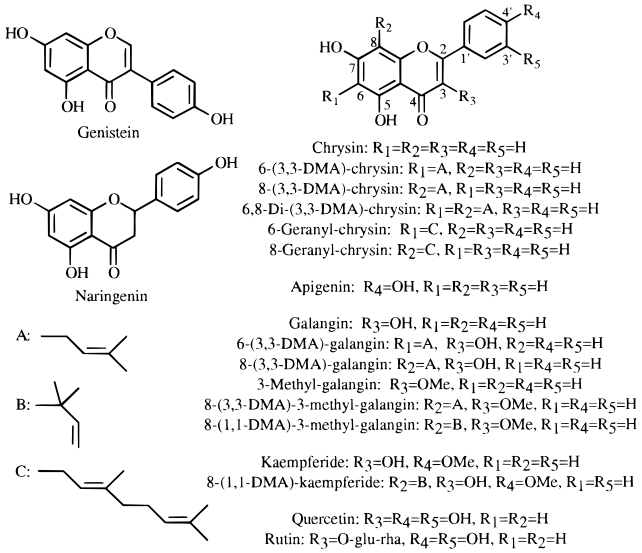

FIGURE 1: Structures of the flavonoids that were studied. The left part shows the isoflavone genistein, the flavanone naringenin, and the different prenyl substituents: 3,3-DMA (A), 1,1-DMA (B), and geranyl (C). The right part shows the different flavones differentiated by their substituents (R_1-R_5).

transfer. This allows the screening of a number of modulators such as flavonoids and the selection of those exhibiting high-affinity binding like prenyl-flavones. Moreover, the Pdr5p-enriched plasma membranes of the mutant strains exhibit ATP and UTP hydrolysis activity (*19*, *21*). They also display a MgATP-dependent rhodamine 6G interaction at the Pdr5p drug-binding site(s), monitored by fluorescence quenching of the probe, that correlates with energy-dependent transport of rhodamine 6G in the mutant yeast cells overexpressing the transporter (*23*).

The work presented here shows that high-affinity binding of prenyl-flavonoids to Pdr5p correlates to strong competitive inhibition of the quenching of rhodamine 6G fluorescence. In contrast, inhibition of ATPase or UTPase activity by the same prenyl-flavonoids requires much higher concentrations and appears to be noncompetitive.

EXPERIMENTAL PROCEDURES

Materials. The 2′(3′)-*N*-methylanthraniloyl (MANT) fluorescent derivative of ATP, MANT-ATP (*24*), and the antiprogestin RU 486 (*25*) were obtained as described previously. Most commercial compounds were from Sigma [ATP, UTP, emetine, vinblastin, paclitaxel, verapamil, daunomycin, cyclosporin A, trifluoperazine, prenylamine, chrysin, quercetin, naringenin, and rutin]. Rhodamine 6G came from British Drug Chemicals or Merck and *n*-dodecyl β-D-maltoside from Boehringer Mannheim. Apigenin, genistein, and kaempferide were from Extrasynthèse (Genay, France), and galangin was from Aldrich. Taxotere was provided by Aventis (Antony and Vitry-Alforville, France), and prenylated derivatives of kaempferide (*26*) and of chrysin and galangin[2] were synthesized as described previously. The structures of the flavonoids studied in the work presented here are shown in Figure 1.

[2] Submitted for publication.

Preparation of Pdr5p-Enriched Membranes. Plasma membranes from the mutant strain AD124567 overexpressing Pdr5p and multideleted in genes encoding not only the Pdr3p regulator but also five ABC transporters [Yor1p, Snq2p, Pdr10p, Pdr11p, and Ycf1p (*20*)] were prepared as previously described for the parental mutant US50-18C (*23*). They were resuspended in 50 mM Hepes/KOH (pH 7.0). Control plasma membranes without Pdr5p were prepared under the same conditions from the mutant strain AD1234567 where the *PDR5* gene was also disrupted (*20*). The protein content was determined by the method of Lowry et al. (*27*) with bovine serum albumin as a standard, and the membranes were aliquoted and frozen in liquid nitrogen before being stored at −70 °C.

Purification of Pdr5p. The multidrug transporter Pdr5p was prepared from plasma membranes of the strain AD124567 by solubilization with 0.1% *n*-dodecyl β-D-maltoside and fractionation by ultracentrifugation on a continuous 6 to 30% sucrose gradient containing 1 mM ATP and 1 mM ethylenediaminetetraacetic acid (EDTA), as previously described for strain US50-D2 (*19*). A parallel control experiment without Pdr5p was performed under the same conditions with strain AD1234567 with a disrupted *PDR5* gene. The fractions were assayed for protein content, dialyzed against 10 mM Tris-HCl (pH 7.5) containing 12% sucrose, 0.02% *n*-dodecyl β-D-maltoside, and 1 mM EDTA, and kept frozen at −20 °C.

ATPase and UTPase Assays. Nucleoside triphosphate hydrolysis was assessed by incubating Pdr5p-enriched membranes (10−20 μg of protein), or the same volumes of control membranes, at 35 °C in 500 μL of 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid], at 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 ATP or UTP and excess $MgCl_2$ providing 1 mM free Mg^{2+}. After 5−15 min, the reaction was stopped with trichloroacetic acid, and the amount of released inorganic phosphate was titrated colorimetrically (*28*) as previously described (*19*−*21*). When flavonoid inhibition was studied, stock solutions in dimethyl sulfoxide were added, up to a 2−4% (v/v) final concentration.

Fluorescence Experiments. Intrinsic-fluorescence experiments with the purified Pdr5p transporter were performed at 25.0 ± 0.1 °C using an SLM-Aminco 8000C spectrofluorimeter with spectral bandwidths of 2 and 4 nm for excitation and emission, respectively. The measurements were corrected for the wavelength dependence on the exciting-light intensity, for buffer fluorescence, and for dilution, as previously described for other proteins (*14*, *25*, *29*). Fluorescence measurements were performed by diluting the Pdr5p solution (0.05 μM final concentration, corresponding to a concentration of 1 μM in tryptophan residues), or the same volume of the control fraction without Pdr5p, in 1.2 mL of 10 mM Tris-HCl (pH 7.5) containing 12% (w/v) sucrose, 0.02% (w/v) *n*-dodecyl β-D-maltoside, and either 1 mM EDTA or 5 mM $MgCl_2$. The emission fluorescence spectra were recorded from 310 to 360 nm (or to 530 nm when MANT-ATP was present) upon excitation at 295 nm. For quenching studies, successive aliquots of effector solutions in dimethyl sulfoxide were added. Purified Pdr5p fluorescence was first corrected from the contribution of the control without Pdr5p, and then from the ligand inner-filter effect, as measured in parallel experiments using *N*-acetyl-

tryptophanamide. Curve fitting of the concentration-dependent binding of substrates or effectors was performed with the Grafit program (Erithacus Software) as described in detail previously (*14*, *25*, *29*).

When rhodamine 6G fluorescence quenching was studied, Pdr5p-enriched plasma membranes (70−130 μg of protein), or the same volumes of the control membranes without Pdr5p, were incubated at 30 °C in 2−2.2 mL of 50 mM Hepes (pH 7.0) in the presence of the fluorescent probe up to a concentration of 133−144 nM. The excitation was performed at 525 nm and the emission measured at 552 nm with an Aminco-Bowman type 2 spectrofluorimeter. The reaction was initiated by adding equimolar amounts of ATP and $MgCl_2$ (4.4−4.8 mM), after flavonoid addition from a dimethyl sulfoxide solution. The decrease in fluorescence was recorded for 20−40 min, and the linear initial slope was calculated. The $I_{50\%}$ values of rhodamine 6G fluorescence quenching were determined with the Grafit program as previously described (*23*).

RESULTS

Interaction of Purified Pdr5p with Substrates and Effectors. Purified Pdr5p, which contains a total of 21 tryptophan residues (*22*), displayed a characteristic intrinsic-fluorescence spectrum with a maximal emission wavelength at 324 nm upon excitation at 295 nm (Figure 2A). This indicated that the tryptophan environment was mostly hydrophobic. Addition of increasing concentrations of MANT-ATP, a fluorescent ATP analogue shown to bind with a high affinity to other ABC transporters (*14*, *15*, *24*, *25*), produced a high quenching of Pdr5p intrinsic fluorescence that was accompanied by the appearance of a new peak of emission fluorescence centered around 430 nm. This was indicative of fluorescence resonance-energy transfer between Pdr5p tryptophan residues and bound MANT-ATP. An apparent K_d value of 5.2 ± 0.8 μM was estimated, while the maximal quenching of intrinsic fluorescence was 61.3 ± 2.4%. The binding affinity was Mg^{2+}-dependent since a 2-fold increase was observed upon addition of 5 mM $MgCl_2$ (K_d = 2.7 ± 0.9 μM).

Table 1 shows a similar Mg^{2+} dependence with the natural substrate ATP, since the K_d value changed from 450 ± 160 to 200 ± 50 μM, although a much lower maximal quenching (14−22%) was produced. The interaction of a number of other known drug substrates or modulators was monitored by the same experimental approach. The K_d values toward drugs ranged from 0.27 ± 0.06 μM for emetine to 17.3 ± 2.9 μM for daunomycin, whereas those toward modulators ranged from 1.1 ± 0.2 μM for cyclosporin A to 6.2 ± 0.8 μM for RU 486 which bound with a much higher affinity than progesterone (not shown here). Hoechst 33342 also bound, with a K_d of 3.3 ± 0.4 μM (data not shown). All these K_d values were comparable to those previously reported, from other approaches, for membrane-inserted Pdr5p (*23*) and for cancer cell P-glycoprotein (*16*, *30*−*34*), suggesting that the drug-binding properties of purified Pdr5p were conserved.

A number of flavonoids (see Figure 1) were checked in a similar way, and found to bind Pdr5p differently. The structure of 6-(3,3-DMA)galangin and its concentration-dependent binding are illustrated in Figure 2B. Table 2 shows

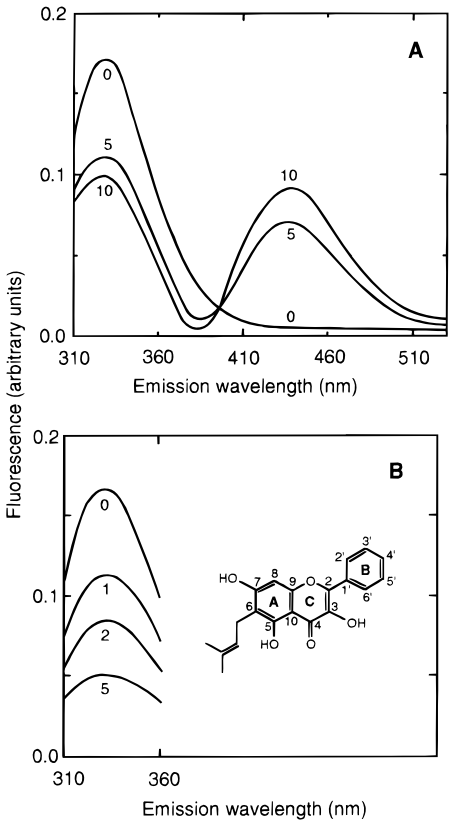

FIGURE 2: Direct interaction of purified Pdr5p with effectors as monitored by changes in fluorescence. (A) Fluorescence resonance-energy transfer with MANT-ATP. Purified Pdr5p (at a tryptophan final concentration of 1 μM), or the same volume of the control preparation without Pdr5p, was incubated at 25 ± 0.1 °C with the indicated micromolar concentrations of MANT-ATP, in 1.2 mL of fluorescence buffer consisting of 10 mM Tris-HCl (pH 7.5), 12% (w/v) sucrose, 1 mM EDTA, and 0.02% (w/v) *n*-dodecyl β-D-maltoside. Upon excitation at 295 nm, the emission fluorescence spectra were recorded and corrected for control assays performed under the same conditions but in the absence of protein. The differential spectra of Pdr5p corrected from the control are shown. (B) Quenching of intrinsic fluorescence by a prenyl-flavonoid. Pdr5p, or the control without Pdr5p, was incubated as described for panel A with the indicated micromolar concentrations of 6-(3,3-DMA)galangin whose structure is shown.

that the flavones, with ring B branched at position 2 of ring C and a 2,3 double bond (apigenin, galangin, and quercetin), bound with better affinity (K_d = 6.3−13 μM) than the corresponding isoflavone, with ring B branched at position 3 (K_d = 30.0 ± 7.8 μM), and flavanone, with a reduced 2,3 bond (K_d = 26.5 ± 5.1 μM), whereas hydrophilic glucorhamnosylation in rutin almost completely abolished the interaction. Hydroxylation at position 3 of ring C improved the binding affinity of galangin as compared to that of chrysin. Hydroxylation at positions 4′ and 3′ (quercetin) of ring B reduced the binding affinity, while methoxylation at position 4′ (kaempferide) produced a positive effect. A further increase in affinity was observed with flavonoids which were C-isoprenylated at position 6 or 8 of ring A, such as dimethylallyl (DMA) derivatives of either galangin, kaempferide, or chrysin that bound with K_d values in the micromolar range [as shown in Figure 2B for 6-(3,3-DMA)-galangin].

Interference of Flavonoid Binding toward Nucleotide Hydrolysis. Pdr5p-enriched membranes are known to hy-

Table 1: Interaction of Purified Pdr5p with Substrates and Known Modulators[a]

substrate or modulator	K_d (μM)	ΔF_{max} (%)
ATP	200 ± 50	21.9 ± 1.8
ATP (Mg^{2+} omitted)	450 ± 160	13.9 ± 1.1
emetine	0.27 ± 0.06	79.2 ± 3.7
vinblastine	0.57 ± 0.42	26.8 ± 3.5
paclitaxel	1.07 ± 0.33	53.0 ± 3.8
daunomycin	17.3 ± 2.9	79.2 ± 3.7
cyclosporin A	1.1 ± 0.2	47.9 ± 2.8
trifluoperazin	3.6 ± 0.3	93.4 ± 2.0
prenylamine	3.7 ± 0.7	77.2 ± 3.8
RU 486	6.2 ± 0.8	92.9 ± 3.5

[a] Purified Pdr5p, or the control fraction without Pdr5p, was incubated in the fluorescence buffer described in the legend of Figure 2 supplemented with 5 mM $MgCl_2$, in the presence of the indicated substrate or modulator at increasing concentrations. The quenching of Pdr5p intrinsic fluorescence was assessed by integration of the fluorescence spectrum between 310 and 360 nm. It was subtracted from the control without Pdr5p and corrected for buffer contribution and the inner-filter effect determined with *N*-acetyltryptophanamide. Analysis of the data with the Grafit program gave the values of the dissociation constant (K_d), and of the maximal quenching of fluorescence by reference to the emission fluorescence in the absence of effector (ΔF_{max}).

Table 2: Differential Interaction of Purified Pdr5p with Flavonoids[a]

flavonoid	K_d (μM)	ΔF_{max} (%)
apigenin (5,7,4′-trihydroxyflavone)	7.7 ± 1.4	101.5 ± 5.2
genistein (5,7,4′-trihydroxyisoflavone)	30.0 ± 7.8	68.4 ± 8.2
naringenin (5,7,4′-trihydroxyflavanone)	26.5 ± 5.1	101.0 ± 8.3
galangin (3,5,7-trihydroxyflavone)	6.3 ± 0.5	93.1 ± 21
quercetin (3,5,7,3′,4′-pentahydroxyflavone)	13.0 ± 2.9	100.0 ± 6.9
rutin (3-*O*-glucorhamnosylquercetin)	nd[b]	nd
kaempferide (3,5,7-trihydroxy-4′-methoxyflavone)	5.1 ± 0.6	107.1 ± 2.8
chrysin (5,7-dihydroxyflavone)	9.3 ± 1.8	115.7 ± 11.8
3-methylgalangin	6.4 ± 1.1	96.4 ± 7.6
8-(3,3-DMA)galangin	2.2 ± 0.2	91.1 ± 2.9
6-(3,3-DMA)galangin	1.6 ± 0.1	90.5 ± 2.3
8-(1,1-DMA)kaempferide	1.6 ± 0.2	102.3 ± 2.2
8-(3,3-DMA)-3-methylgalangin	2.6 ± 0.3	103.9 ± 3.0
8-(3,3-DMA)chrysin	3.1 ± 0.2	100.1 ± 1.9
6-(3,3-DMA)chrysin	1.1 ± 0.1	52.7 ± 1.5
6,8-di-DMA-chrysin	1.3 ± 0.3	33.6 ± 1.6
8-geranylchrysin	4.6 ± 0.4	99.8 ± 2.5
6-geranylchrysin	3.4 ± 0.5	88.9 ± 4.0

[a] Purified Pdr5p, or the control fraction without Pdr5p, was incubated in the fluorescence buffer of Figure 2 in the presence of increasing concentrations of the indicated flavonoids. The binding parameters of each compound toward Pdr5p were determined by quenching of intrinsic fluorescence as described in Table 1. [b] nd, not accurately determinable, as due to an excessively low level of quenching.

drolyze both UTP and ATP (*21*). The maximal rates measured here at pH 7.5 were 0.86 ± 0.09 μmol of UTP hydrolyzed min^{-1} (mg of protein)$^{-1}$ and 1.07 ± 0.24 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$. No UTP hydrolysis and a low value of 0.14 ± 0.04 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$ were measured with control membranes where *PDR5* was disrupted. This low residual ATPase activity indicated a limited contamination, possibly due to Pma1p (*21*). The interference produced by flavonoid binding was studied on both ATPase and UTPase activities of Pdr5p-enriched membranes. Table 3 shows that UTPase activity was always inhibited at lower flavonoid concentrations than ATPase activity; the $I_{50\%}$ values in the latter case could not be accurately measured with quercetin

Table 3: Inhibition of ATPase and UTPase Activities by Flavonoids[a]

flavonoid	$I_{50\%}$ (μM) ATPase	$I_{50\%}$ (μM) UTPase
quercetin	nd[b]	450
8-(1,1-DMA)kaempferide	nd	38.6 ± 5.3
8-(3,3-DMA)-3-methylgalangin	80.4 ± 5.6	14.4 ± 5.1
6-(3,3-DMA)galangin	50 ± 10	4.9 ± 0.8

[a] Pdr5p-enriched membranes, or control membranes without Pdr5p, were incubated with different concentrations of flavonoids and assayed for their ATPase and UTPase activities at pH 7.5 with either 6 mM ATP or UTP and 1 mM excess Mg^{2+}. The rate of hydrolysis was measured by a phosphate-release colorimetric method as described in Experimental Procedures. The $I_{50\%}$ value corresponded to 50% inhibition of the rate obtained in the absence of flavonoid. [b] nd, not accurately determinable, as due to an excessively low level of inhibition.

and 8-(1,1-DMA)kaempferide, as due to an excessively low level of inhibition, and were 6−10-fold higher with 8-DMA-3-methylgalangin and 6-DMA-galangin. The most potent inhibition of UTPase activity was observed with 6-DMA-galangin ($I_{50\%}$ = 4.9 ± 0.8 μM). The level of inhibition was dependent on hydrophobicity as shown with a series of chrysin derivatives in Figure 3A. Whereas unsubstituted chrysin, up to 200 μM, did not inhibit UTPase activity, the addition of increasingly hydrophobic substituents progressively increased the inhibition as follows: 8-geranyl > 6-geranyl > 6,8-di-DMA > 6-DMA > 7-*O*-DMA > H.

When increasing concentrations of the most efficient flavonoid, 6-(3,3-DMA)galangin, were used in the presence of increasing substrate concentrations, double-recriprocal plots indicated a noncompetitive inhibition toward either UTP (panel B) or ATP (panel C), with respective K_i values of 5.4 ± 0.1 and 62 ± 21 μM.

Interference of Flavonoid Binding on the Quenching of Rhodamine 6G Fluorescence. Pdr5p-enriched plasma membranes were shown to catalyze a MgATP-dependent interaction with rhodamine 6G, monitored by quenching of the probe fluorescence, which was inhibited by micromolar concentrations of drugs or modulators (*23*). In contrast, no change in rhodamine 6G fluorescence was produced by control membranes lacking Pdr5p. Table 4 shows that the most potent inhibitions were observed with Taxotere (docetaxel), a hemisynthetic taxoid from european yew (*33*), and with the antiprogestin RU 486, giving submicromolar $I_{50\%}$ values at 133 nM rhodamine 6G. Tested flavonoids also inhibited the quenching of fluorescence, the potency strongly depending on the substituents. The $I_{50\%}$ values varied from 8.5 to 1.3 μM for nonprenylated flavonoids (chrysin, 3-methylgalangin, galangin, quercetin, or kaempferide) down to values from 0.94 to 0.24 μM for prenylated derivatives of chrysin and galangin, respectively. Thus, prenylation increased the extent of inhibition by about 1 order of magnitude, the best compound being 6-(3,3-DMA)galangin. Here also, the effect was dependent on substituent hydrophobicity since di-DMA and geranyl derivatives of chrysin were at least 2-fold more potent than the mono-DMA derivative.

When flavonoids were used in the presence of various rhodamine 6G concentrations, a competitive inhibition of fluorescence quenching was always observed, as illustrated in Figure 4 where the apparent K_m for rhodamine 6G (42.9 ± 14.2 nM) increased in the presence of micromolar

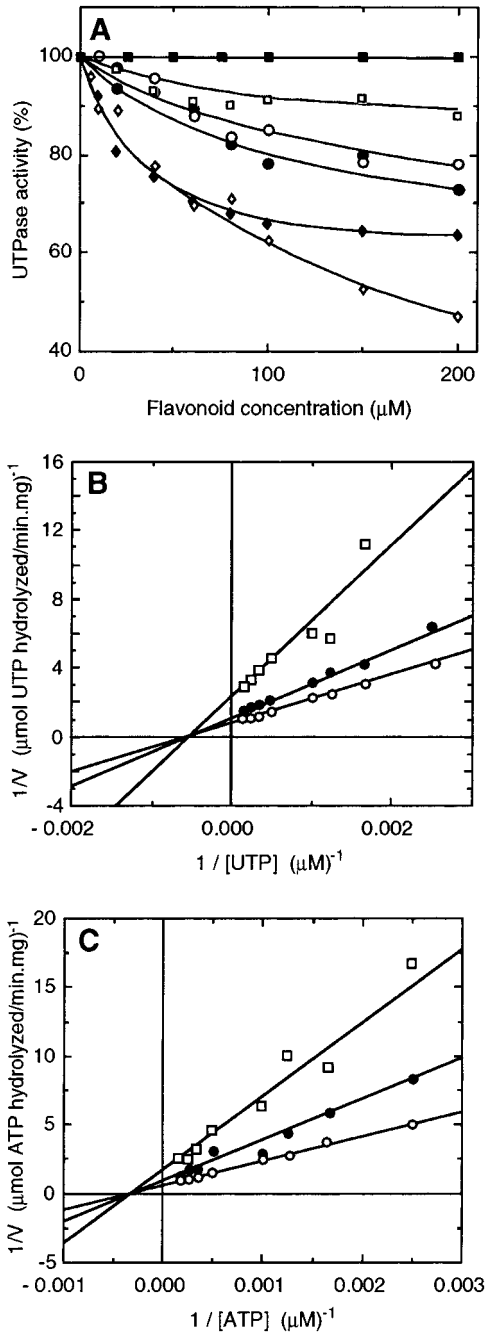

FIGURE 3: Effects of substituent hydrophobicity on flavonoid efficiency in inhibiting nucleotide hydrolysis, and the type of inhibition. Pdr5p-enriched membranes were incubated with increasing concentrations of flavonoids and assayed for hydrolytic activity in the presence of different concentrations of either MgUTP or MgATP. (A) Inhibition of the hydrolysis of 6 mM MgUTP, assessed as described in Table 3, by increasing concentrations of the following chrysin derivatives: unsubstituted chrysin (■), 7-*O*-DMA-chrysin (□), 6-DMA-chrysin (○), 6,8-di-DMA-chrysin (●) , 6-geranylchrysin (♦), or 8-geranylchrysin (◊). (B) Double-reciprocal plots of UTPase activity, at substrate concentrations ranging from 0.4 to 6 mM, in the absence of 6-(3,3-DMA)galangin (○) or in the presence of a concentration of 2.5 (●) or 10 μM (□). (C) Double-reciprocal plots of ATPase activity without 6-(3,3-DMA)galangin (○) or at a concentration of 25 (●) or 50 μM (□).

concentrations of flavonoids. The K_i values determined for chrysin (panel A), galangin (panel B), and 6-(3,3-DMA)-galangin (panel C) were 2.7 ± 0.5, 1.1 ± 0.1, and 0.18 ± 0.05 μM, respectively.

Table 4: Inhibition of Energy-Dependent Quenching of Rhodamine 6G Fluorescence[a]

substrate or modulator	$I_{50\%}$ (μM)
paclitaxel	2.8 ± 0.4
Taxotere	0.99 ± 0.14
verapamil	1.7 ± 0.1
RU 486	0.53 ± 0.04
quercetin	1.5 ± 0.01
kaempferide	1.3 ± 0.1
8-(1,1-DMA)kaempferide	0.33 ± 0.04
galangin	2.2 ± 0.1
8-(1,1-DMA)galangin	0.39 ± 0.01
8-(3,3-DMA)galangin	0.57 ± 0.03
6-(3,3-DMA)galangin	0.24 ± 0.03
3-methylgalangin	5.9 ± 0.2
8-(1,1-DMA)-3-methylgalangin	0.49 ± 0.02
8-(3,3-DMA)-3-methylgalangin	0.67 ± 0.05
chrysin	8.5 ± 0.6
8-(3,3-DMA)chrysin	0.94 ± 0.05
6,8-di-DMA-chrysin	0.39 ± 0.01
6-geranylchrysin	0.43 ± 0.05

[a] Pdr5p-enriched membranes, or control membranes without Pdr5p, were incubated with increasing concentrations of either drug, modulator, or flavonoid and assayed for energy-dependent fluorescence quenching of 133 nM rhodamine 6G in the presence of ATP and $MgCl_2$. The $I_{50\%}$ values corresponded to effector concentrations producing 50% inhibition of the initial slope of fluorescence quenching without effector.

DISCUSSION

This paper reports the following new properties of prenylflavonoids. (i) They bind with high affinity to the purified yeast multidrug transporter Pdr5p. (ii) They inhibit competitively the rhodamine 6G binding to Pdr5p within isolated plasma membranes. (iii) They exert a low-affinity and noncompetitive inhibition of ATP or UTP hydrolysis carried out by membrane-bound Pdr5p.

Purified Pdr5p and High-Affinity Binding of Prenyl-Flavonoids. The high level of Pdr5p overexpression in the *pdr1-3* mutant of the yeast *Saccharomyces cerevisiae* allows the purification of sufficiently high amounts of the transporter for biophysical studies, such as fluorescence experiments to monitor its direct interactions with effectors. The purification upon detergent solubilization and ultracentrifugation on sucrose gradient is facilitated here by the absence of other ABC transporters with similar molecular weights, by using strain AD124567 (*20*). Moreover, an accurate control is provided by strain AD1234567 where the Pdr5p-encoding gene is also disrupted (*20*). Experiments with a number of different ligands suggest that purified Pdr5p retains its binding properties. (i) The binding affinity of both ATP and its fluorescent derivative, MANT-ATP, is magnesium-dependent. (ii) Cytotoxic drugs known as characteristic transported substrates of cancer cell P-glycoprotein, such as paclitaxel, Taxotere, vinblastin, and daunomycin bind to purified Pdr5p at micromolar concentrations similar to those used from inhibition studies of drug interaction with the same transporter (*23*) or with cancer cell P-glycoprotein (*30*, *31*, *34*). (iii) The hydrophobic antiprogestin RU 486 binds with a much higher affinity than progesterone, in agreement with the higher-affinity binding to a cytosolic domain of either P-glycoprotein (*25*) or *L. tropica* multidrug transporter (*15*), or to purified P-glycoprotein (*25*), and with differential modulation of MDR cancer cells (*35*, *36*).

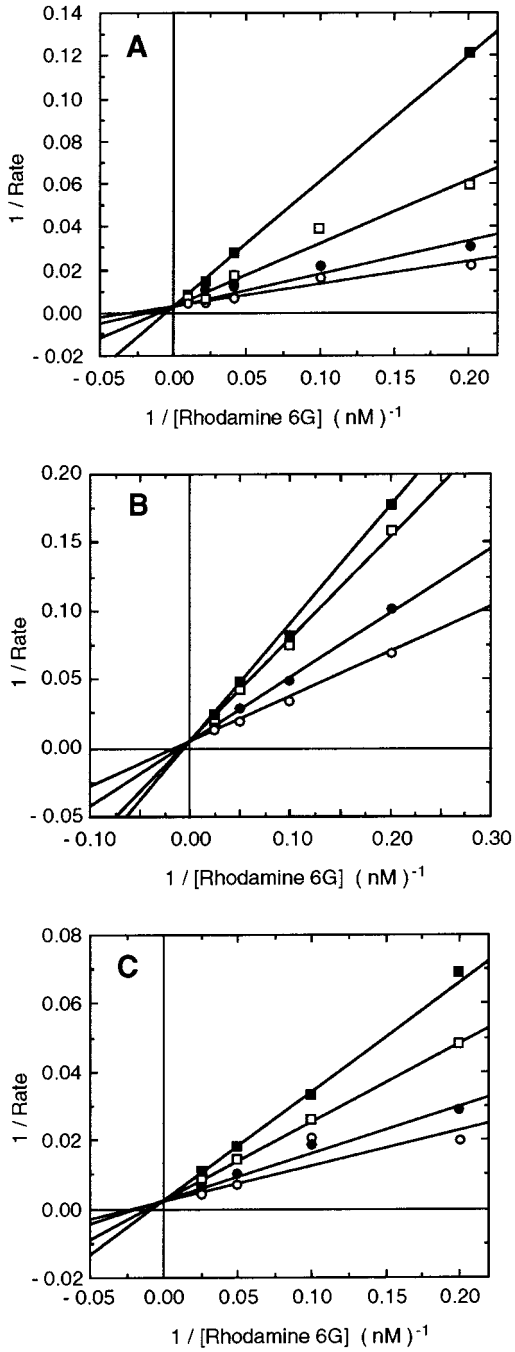

FIGURE 4: Competitive inhibition by flavonoids of quenching of rhodamine 6G fluorescence. Pdr5p-enriched membranes were incubated with increasing flavonoid concentrations and assayed, as described in Table 4, for MgATP-dependent fluorescence quenching of rhodamine 6G at concentrations ranging from 5 to 133 nM. (A) Double-reciprocal plots obtained with chrysin at 0 (○), 2.5 (●), 5 (□), or 10 μM (■). (B) Double-reciprocal plots obtained with galangin at 0 (○), 0.5 (●), 1 (□), or 2 μM (■). (C) Double-reciprocal plots obtained with 6-(3,3-DMA)galangin at 0 (○), 0.05 (●), 0.1 (□), or 0.2 μM (■).

Purified Pdr5p is therefore a material of special interest for screening a series of flavonoids. It exhibits the same preference for flavones over isoflavones and flavanones, and for hydrophobic over hydrophilic derivatives as cancer cell P-glycoprotein (*14*) and the P-glycoprotein-like multidrug transporter from *L. tropica* (*15*). Together with the above results, this suggests that, despite a different domain arrangement in the sequence where the nucleotide-binding domains are preceding instead of following the transmembrane ones (*22*), yeast Pdr5p displays substrate-binding properties similar to those of P-glycoprotein probably due to the same three-dimensional organization. The requirement for a hydroxyl group at position 3 of ring C as for the tyrosine kinase Hck (*11*) and H^+,K^+-ATPase (*37*), in addition to the hydroxyl at position 5 of ring A (*14*) and the ketone at position 4 of ring C as in CDK2 (*10*), could favor interaction at the ATP-binding site. On the other hand, the increase in affinity by methoxylation at position 4′ of ring B and by prenylation at position 6 and/or 8 of ring A suggests a strengthened interaction at the hydrophobic drug and modulator binding site(s). Therefore, two different types of substitution both produce an increase in binding affinity toward purified Pdr5p, but the possibility that the flavonoid could then bind differently and occupy distinct binding sites cannot be excluded.

High-Affinity Competitive Inhibition of Drug Interaction. The plasma membranes from the new mutant strain used here, AD124567 (*20*), exhibit a better affinity for rhodamine 6G than those from the parental mutant strain US50-18C, with an apparent K_m value of 42.9 nM as compared to 144 nM (*23*); this might be related to the fact that they are highly and specifically enriched with Pdr5p since the genes encoding the other ABC transporters with similar molecular weights have been disrupted. Therefore, the presence of other ABC transporters, such as Snq2p, Yor1p, and Ycf1p in the US50-18C membranes, which did not produce any quenching of rhodamine 6G fluorescence (*23*), could however bind some amount of the probe and therefore increase its apparent K_m for Pdr5p.

The experiments concerning rhodamine 6G interaction with these Pdr5p-enriched membranes strongly suggest that inhibitory flavonoids bind to the drug-binding site. (i) All the flavonoids tested [chrysin, galangin, and 6-(3,3-DMA)-galangin] produce a competitive inhibition of rhodamine 6G fluorescence quenching with K_i values ranging from 2.7 to 0.18 μM, which are significantly lower than the corresponding $I_{50\%}$ values determined at a nearly saturating rhodamine 6G concentration. (ii) Such a flavonoid concentration range does not affect ATPase activity. (iii) The K_i and $I_{50\%}$ values for inhibition of membrane Pdr5p-mediated quenching of rhodamine 6G fluorescence are comparable to the K_d values determined for direct binding to the purified transporter. These values are even lower in purified Pdr5p for the hydrophobic prenyl-flavonoids, which might be due to some interference of the detergent, required at a minimal 0.02% concentration to maintain solubility of the purified protein; a similar difference is also observed for the hydrophobic antiprogestin RU 486.

Lower-Level Noncompetitive Inhibition of Hydrolytic Activity. All the tested flavonoids require high concentrations to inhibit ATPase activity. For 6-(3,3-DMA)galangin, the K_i is 200-fold higher (50−62 μM) than for quenching of rhodamine 6G fluorescence (0.18−0.24 μM). This indicates that the competitive inhibition produced by submicromolar concentrations of flavonoid on rhodamine 6G interaction is indeed due to direct binding to the drug-binding site(s) and not to inhibition of ATP hydrolysis providing the energy-driving force. Nonprenylated flavonoids, such as quercetin, kaempferide, galangin, 3-methylgalangin, and chrysin, also appear to bind to Pdr5p drug-binding site(s) despite a lower

affinity. The concentration range is comparable to that required in purified reconstituted P-glycoprotein for quercetin interaction, assumed to occur at a drug-binding site characterized by Hoechst 33342 binding (*33*).

UTPase activity is more sensitive than ATPase activity to flavonoid inhibition, but in any case, the concentration range remains relatively high as compared to the effects produced on drug interaction. In addition, the inhibition appears to be noncompetitive for prenyl-flavonoids, as evidenced from both the horizontal axis intercept in double-reciprocal plots of inhibition kinetics and the fact that the K_i values are not significanly different from the $I_{50\%}$ values. This strongly suggests that binding occurs outside the nucleotide-binding site(s). Two alternative explanations could be considered for this second flavonoid-binding site which might either (i) be a second nonequivalent drug-binding site, as observed for other effectors with P-glycoprotein (*33*, *38*), or (ii) be located in the hydrophobic region detected on the cytosolic domains of the transporter through interaction with steroid modulators (progesterone, RU 486) as characterized by using purified recombinant NBDs of P-glycoprotein (*14*, *25*) and the *L. tropica* multidrug transporter (*15*). Flavonoids and prenylated derivatives were indeed found to interact at such a hydrophobic region (*14*, *15*). In that case, the inhibition of ATP or UTP hydrolysis might be due to steric hindrance, or to prevention of a conformational change related to signal transduction from nucleotide hydrolysis to the drug-binding site(s).

The higher sensitivity to any effector of UTPase as compared to ATPase activity might be due (i) to some contamination of Pma1p which hydrolyzes ATP but not UTP (*39*) or (ii) more likely to different mechanisms of ATP and UTP hydrolysis by Pdr5p, possibly related to differential binding stoichiometry, especially since Pdr5p contains a nucleotide-binding site with partially degenerated consensus sequences (*22*), or to different conformational changes induced by nucleotide hydrolysis for signal transduction.

For nonprenylated flavonoids (chrysin, galangin, and quercetin), the low level of inhibition of nucleotide hydrolysis did not allow an accurate determination of the type of inhibition. Therefore, the possibility that galangin and quercetin binding might partly overlap the nucleotide-binding site could not be excluded as this was indeed shown for kaempferide with the recombinant nucleotide-binding domain of P-glycoprotein (*14*) and proposed for quercetin with reconstituted P-glycoprotein (*16*).

The overall results indicate that prenyl-flavonoids, especially 6-(3,3-DMA)galangin, constitute potent inhibitors of the yeast Pdr5p multidrug transporter. Additional work is in progress to further characterize the lower-affinity binding site responsible for inhibition of nucleotide hydrolysis, whose mechanism has to be clarified.

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