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Flavonoids: A class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein

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ABSTRACT **A hexahistidine-tagged C-terminal nucleotide-binding domain (H_6-NBD2) from mouse P-glycoprotein was designed, overexpressed, and purified as a highly soluble recombinant protein. Intrinsic fluorescence of its single tryptophan residue allowed monitoring of high-affinity binding of 2′(3′)-*N*-methylanthraniloyl-ATP (MANT-ATP), a fluorescent ATP derivative that induces a marked quenching correlated to fluorescence resonance-energy transfer. H_6-NBD2 also bound all flavonoids known to modulate the multidrug resistance phenotype of P-glycoprotein-positive cancer cells, with similar affinities and relative efficiencies. Flavones (like quercetin or apigenin) bound more strongly than flavanones (naringenin), isoflavones (genistein), or glycosylated derivatives (rutin). Kaempferide, a 4′-methoxy 3,5,7-trihydroxy flavone, was even more reactive and induced a complete quenching of H_6-NBD2 intrinsic fluorescence. Kaempferide binding was partly prevented by preincubation with ATP, or partly displaced upon ATP addition. Interestingly, kaempferide was also able to partly prevent the binding of the antiprogestin RU 486 to a hydrophobic region similar to that recently found, close to the ATP site, in the N-terminal cytosolic domain. Conversely, RU 486 partly prevented kaempferide binding, the effect being additive to the partial prevention by ATP. Furthermore, MANT-ATP binding, which occurred at the ATP site and extended to the vicinal steroid-interacting hydrophobic region, was completely prevented or displaced by kaempferide. All results indicate that flavonoids constitute a new class of modulators with bifunctional interactions at vicinal ATP-binding site and steroid-interacting region within a cytosolic domain of P-glycoprotein.**

Multidrug resistance (MDR) of cancer cells is often associated with overexpression of P-glycoprotein, a plasma membrane transporter that extrudes chemotherapeutic drugs by using ATP hydrolysis as the energy source (1, 2). The MDR1 P-glycoprotein and its MDR2 isoform are encoded by two *mdr* genes in human, whereas three genes are present in mouse: *mdr*1 (or *mdr*1b), *mdr*2, and *mdr*3 (or *mdr*1a). Both *mdr*1 and *mdr*3 can convey cellular MDR in transfection studies as opposed to *mdr*2. The *mdr*2 product appears to be involved in selective translocation of phosphatidylcholine (3), whereas *mdr*1 P-glycoprotein may act on a very broad range of short-chain phospholipids (4), in addition to many other amphipathic compounds. P-glycoprotein is also able to bind a number of modulators, most of which are transported (5). Interestingly, steroids are either transported or not, depending on their hydrophobicity. Hydroxylated steroids, including cortisol, dexamethasone, aldosterone, or corticosterone, can be efluxed by P-glycoprotein (6, 7), whereas more hydrophobic ones such as progesterone and antiprogestin RU 486 are not transported and behave as efficient modulators of cellular MDR by inhibiting anticancer drug efflux (6, 8, 9). The modulatory efficiency of hydrophobic steroids could be recently correlated to their high-affinity binding to N-terminal cytosolic domain, close to the ATP-binding site (10). Flavonoids have also been described as modulators, but contradictory effects were reported with different multidrug-resistant cell lines: quercetin, kaempferol, and galangin were found to increase adriamycin efflux from HCT-15 colon cells (11), whereas quercetin and a methoxylated derivative inhibited rhodamine 123 efflux and reverted MDR in MCF-7 breast cells (12). Quercetin was indeed shown to bind to purified P-glycoprotein and to efficiently inhibit its activity (13).

Structural analysis of the P-glycoprotein sequence identified a polypeptide of 1,276 amino acids in mice (14), constituted of two homologous halves, each containing six membrane-spanning α-helices (15, 16), and a cytosolic nucleotide-binding domain (NBD) with characteristic Walker motifs A and B (17); both halves are separated by a central linker region with several putative phosphorylation sites for protein kinase C (18). A recent low-resolution structure for P-glycoprotein indicated the existence of a large hydrophilic membrane pore with a probably essential gating ensured by the cytosolic domains (19).

An alternative approach to studying the properties of cytosolic domains of the transporter and their interactions with ligands is to work with recombinant cytosolic domains, predicted to be soluble, after overexpression in bacteria and purification. The C-terminal NBD2 domain was obtained in fusion with either glutathione *S*-transferase (GST) (20) or maltose-binding protein (21), but its separation from the fusion protein gave an unstable domain with low solubility (20). The N-terminal NBD1 domain was produced as a hexahistidine-tagged protein of varying length, the shortest protein corresponding to segment 395–581 being the most soluble (22). However, an extended NBD1 domain including a tryptophan residue allowed the monitoring of a hydrophobic region, interacting with modulator steroids like RU 486, located in close proximity to the ATP-binding site (10).

Because flavonoids are known to also bind to GST (23), the aim of this work was to generate a soluble and stable hexahistidine-tagged NBD2, as short as H_6-NBD1. Its single tryptophan at

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Abbreviations: HECAMEG, 6-*O*-(*N*-heptylcarbamoyl)-methyl-α-D-glucopyranoside; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MANT-ATP, 2′(3′)-*N*-methylanthraniloyl-ATP; MDR, multidrug resistance; NBD, nucleotide-binding domain; NBD1, N-terminal NBD (from Asn-395 to Thr-581); NBD2, C-terminal NBD (from Thr-1044 to Thr-1224); GST, glutathione *S*-transferase.

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position 1106 was used as an intrinsic probe to monitor flavonoid binding, which induced a high quenching of fluorescence and antagonized the binding of both nucleotides and RU 486. The results show that flavonoids behave as bifunctional modulators that overlap vicinal binding sites for ATP and RU 486 within a cytosolic domain of P-glycoprotein.

EXPERIMENTAL PROCEDURES

Materials. The oligonucleotide primers were purchased from Bioprobe Systems (Montreuil-sous-Bois, France) and the nickel-nitrilotriacetic acid agarose gel was from Qiagen S.A. (Courtaboeuf, France). 2′(3′)-*N*-methylanthraniloyl-ATP (MANT-ATP) was obtained as described (22). ATP, HECAMEG [6-*O*-(*N*-heptylcarbamoyl)-methyl-α-D-glucopyranoside], and IPTG (isopropyl-1-thio-β-D-galactopyranoside) were purchased from Boehringer Mannheim. *N*-acetyltryptophanamide, imidazole, dimethyl sulfoxide, chrysin, flavone, quercetin, and rutin came from Sigma. The other flavonoids were from either Aldrich (7-hydroxyflavone and galangin) or Extrasynthèse (Genay, France) (apigenin, 3-hydroxyflavone, genistein, kaempferide, and kaempferol). The antiprogestin RU 486 was obtained as described previously (10).

Construction of the Overexpression Vector. The cDNA encoding a domain corresponding to segment Thr^{1044}–Thr^{1224} of NBD2 was amplified by PCR using the recombinant plasmid encoding GST-NBD2 (20) as a template and the two following primers: 5′-TATGGATCCACCCGACCCAACATCCCAGTGCTT-3′ and 5′-TATAAGCTTCAGGTGCGGCCTTCCCTGGCTTT-3′.

After restriction by *Bam*HI and *Hin*dIII enzymes, the 557-bp amplified cDNA was digested by endonucleases and ligated into the corresponding sites of linearized pQE-30 plasmid (Qiagen). *Escherichia coli* JM109 cells [*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17, (r_K-, m_K+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^qZ Δ M15]] were transformed with the ligation product and grown on agar plates supplemented with ampicillin (50 μg/ml).

Overexpression and Purification of H_6-NBD2. *E. coli* cells harboring the appropriate recombinant plasmid were grown at 37°C in Luria–Bertani medium [1% (wt/vol) bacto-tryptone/0.5% (wt/vol) yeast extract/1% (wt/vol) NaCl at pH 7.5 containing 50 μg ampicillin/ml] until the absorbance at 600 nm reached 0.7 unit. Expression of the recombinant domain was induced with 2 mM IPTG for 2 hr at 37°C. Cell lysis by French press treatment and purification of recombinant H_6-NBD2 from the soluble fraction by a nickel-nitrilotriacetic acid affinity chromatography were performed as previously described for H_6-NBD1 (22). The fractions eluted with 250 mM imidazole were pooled and dialyzed against 20 mM potassium phosphate, 0.5 M NaCl, 20% glycerol, 0.01% HECAMEG, at pH 6.8 (dialysis buffer). The dialysate (1.9 mg protein per ml) was aliquoted and kept frozen in liquid nitrogen. Protein fractions were analyzed on SDS/12% polyacrylamide gels as described by Laemmli (24). Protein concentration was routinely determined by the method of Bradford (25) with the Coomassie blue Plus Protein Assay Reagent kit from Pierce. As also observed for a longer NBD2 domain, either fused to GST or isolated after thrombin cleavage (20), the H_6-NBD2 domain exhibited very low, if any, ATPase activity.

Fluorescence. Experiments were performed by using a SLM-Aminco 8000C spectrofluorometer with spectral bandwidths of 2 and 4 nm, respectively, for excitation and emission. The measurements were corrected for wavelength dependence on the excitation-light intensity by using rhodamine B in the reference channel. All spectra were corrected for the Raman effect of buffer and for dilution.

Fluorescence measurements were performed after dilution of H_6-NBD2 (1.1–6 μM final concentration) and equilibration for 60 min at 25.0 ± 0.1°C in 1.2 ml of dialysis buffer, described above, at pH 6.8, in the presence of increasing concentrations of nucleotides (ATP or MANT-ATP) or dimethyl sulfoxide solutions of either flavonoids or RU 486. Tryptophan-intrinsic fluorescence of H_6-NBD2 was measured by scanning emission in the range 310–380 nm upon excitation at 295 nm. The binding of MANT-ATP, flavonoids, or RU 486 was monitored by the quenching of emission fluorescence produced by addition of increasing concentrations of ligand; corrections for innerfilter effect and dimethyl sulfoxide (up to 2% final concentration) were determined under the same conditions by using *N*-acetyltryptophanamide. Curve fitting of ligand binding related to fluorescence decrease was performed with GRAFIT (Erithacus software) as described (26). Fluorescence resonance-energy transfer between tryptophan-1106 of H_6-NBD2 and bound MANT-ATP was monitored by the appearance of a fluorescence emission peak between 400 and 530 nm, characteristic of bound nucleotide analogue, upon tryptophan excitation at 295 nm.

Extrinsic fluorescence of MANT-ATP was measured upon excitation at 350 nm. The nucleotide–analogue binding was determined from the differential increase in fluorescence, between 420 and 480 nm, in the presence as compared with the absence of H_6-NBD2. Curve fitting of the concentration-dependent analogue binding related to fluorescence increase was performed with GRAFIT as described previously (26). For kaempferide-dependent prevention of MANT-ATP binding, or displacement of bound MANT-ATP, controls were conducted by using the same concentrations of kaempferide and ATP analogue, but in the absence of protein.

RESULTS

Overexpression and Purification of the Cytosolic H_6-NBD2 Domain from Mouse P-Glycoprotein. Fig. 1*A* shows that the H_6-NBD2 protein, which migrated between the 20- and 29-kDa molecular mass markers, was highly overexpressed upon induction with IPTG (lane 3 as compared with lane 2), and constituted the main component of total bacterial proteins. A high amount of recombinant protein appeared soluble and was recovered in the supernatant from centrifugation at 30,000 × *g* for 30 min (lane 4). It was purified by using a nickel-agarose affinity chromatography; the retained fraction was extensively washed, then eluted with 250 mM imidazole (lane 5), and finally dialyzed in 20 mM potassium phosphate, 0.5 M sodium chloride, 20% glycerol, 0.01% HECAMEG, at pH 6.8. About 5 mg protein of purified domain at about 90 μM could be obtained from a 1-liter culture. H_6-NBD2 strongly reacted with C219 monoclonal antibody, specific for the VQXALD sequence (27), when assayed by immunoblotting (not shown).

The apparent molecular mass of the recombinant domain appeared slightly higher than the theoretical value, 21.2 kDa, as also previously observed for other hexahistidine-tagged proteins (10, 22). The intrinsic fluorescence spectrum of purified H_6-NBD2 indicated that the single tryptophan residue, at position 1106 in P-glycoprotein, exhibited a hydrophobic environment because a low wavelength for maximal emission, 328 nm, was observed upon excitation at 295 nm (Fig. 1*B*). A considerable red shift was produced upon denaturation of the protein by overnight incubation with 6 M guanidine, leading to a maximal emission wavelength of 348–350 nm comparable to that observed with *N*-acetyltryptophanamide (not shown).

Interactions with Nucleotides and Flavonoids. Addition of the fluorescent nucleotide–analogue MANT-ATP to purified H_6-NBD2 produced both a high quenching of intrinsic fluorescence and a concomitant increase in a new fluorescence peak with a maximum at 429 nm (Fig. 1*B*). This was characteristic of fluorescence resonance-energy transfer between tryptophan-1106 and bound MANT-ATP. A hydrophobic environment around the MANT group was revealed by the high blue shift (15 nm) with respect to free MANT-ATP in solution (λ_{max} = 444 nm). The spectral modifications showed a saturating dependence on MANT-ATP concentration: curve fitting with the GRAFIT pro-

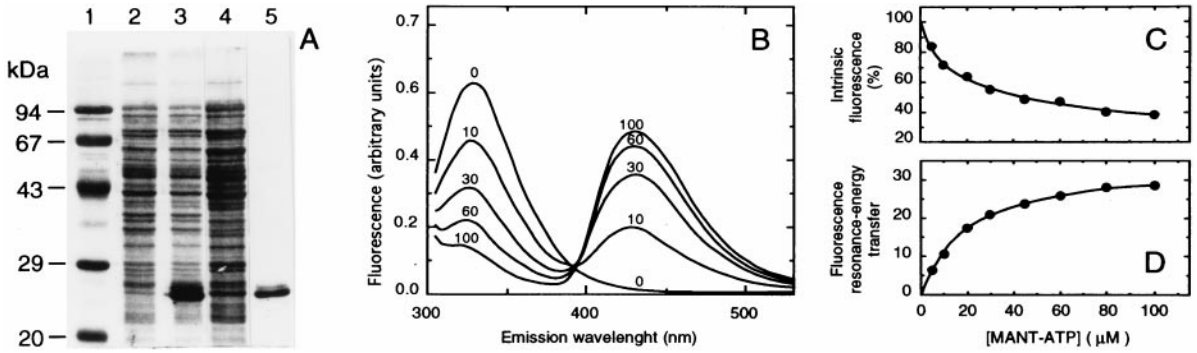

FIG. 1. Purification of the H_6-NBD2 recombinant domain and interaction with MANT-ATP. (*A*) Fractions obtained from a 1-liter culture of *E. coli* cells overexpressing the cDNA encoding H_6-NBD2 were analyzed by SDS/PAGE. Lanes: 1, molecular mass markers corresponding to phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (20 kDa); 2, total bacteria proteins before IPTG induction; 3, total bacteria proteins after IPTG induction; 4, soluble proteins from the supernatant; 5, purified domain obtained after nickel-chelate affinity chromatography. (*B*) Modifications of H_6-NBD2 fluorescence spectrum upon interaction with MANT-ATP. The emission fluorescence of 3.2 μM H_6-NBD2 was recorded after excitation at 295 nm in 1.2 ml of dialysis buffer (see *Experimental Procedures*), at pH 6.8, in the presence of increasing MANT-ATP concentrations from 0 to 100 μM, as indicated on each trace and corrected for buffer contribution. The concentration-dependent binding of MANT-ATP was analyzed by progressive quenching of H_6-NBD2 intrinsic fluorescence, determined by spectral integration from 310 to 380 nm, and corrected for innerfilter effect (*C*), and by the increase in fluorescence resonance-energy transfer between tryptophan and bound MANT-ATP, monitored in the range 400–530 nm (*D*).

gram allowed the determination of very similar dissociation-constant values, 17.7 ± 1.7 μM from quenching of intrinsic fluorescence (Fig. 1*C*) and 18.2 ± 1.1 μM from fluorescence resonance-energy transfer (Fig. 1*D*). A maximal quenching value of 75.5 ± 1.1% was determined from Fig. 1*C*.

Incubation of H_6-NBD2 with various flavonoids also led to marked quenching of the domain intrinsic fluorescence, the efficiency being strongly dependent on the class of the flavonoid (Fig. 2*A*). Quercetin, a 3,5,7,3′,4′-pentahydroxy flavone (Fig. 2*B*), which was previously reported to bind to purified P-glycoprotein and to inhibit its ATP-dependent drug efflux activity (13), is shown here to bind strongly to purified H_6-NBD2 by inducing a complete quenching of intrinsic fluorescence, with an $I_{50\%}$ value of 7 μM. A similar behavior was observed with the other flavone, apigenin, a 5,7,4′-trihydroxy flavone. In contrast, much lower affinities were obtained for naringenin, a flavanone derivative of apigenin, where the 2,3-double bond is reduced ($I_{50\%}$ = 28.5 μM), and for the isoflavone genistein ($I_{50\%}$ = 37 μM), where the ring *b* (compare with Fig. 2*B*) is branched at position 3, instead of position 2 in apigenin. A very low affinity interaction was observed for rutin, a highly hydrophilic, 3-*O*-glucorhamnosyl, derivative of quercetin.

Table 1 shows that the presence of a discrete number of hydroxyl groups on rings *a* and *c* increased the affinity for quenching at pH 6.8 with respect to unsubstituted flavone (K_d = 34.1 ± 11.5 μM). Although no significant effect was produced by hydroxylation at position 7 (K_d = 34.91 ± 4.5 μM), a marked increase in affinity for quenching was observed by hydroxylation at positions 3 (K_d = 10.1 ± 2.5 μM) and 5 (K_d = 8.9 ± 0.3 μM for chrysin). The interaction with galangin was even better (K_d = 5.3 ± 0.1 μM), indicating at least partial additivity of the effects. The importance of a hydroxyl group at position 3 was confirmed by the observation that its substitution by a methoxyl group in 3-methylgalangin markedly increased the apparent K_d value (not shown here). However, further addition of a hydroxyl group at position 4′ of ring *b* produced a negative effect when comparing kaempferol to galangin. In contrast, the presence of a hydrophobic methoxyl group in kaempferide increased the affinity for quenching (K_d = 4.5 ± 0.2 μM) as compared with kaempferol and galangin. The interaction appeared strongly dependent on pH because an apparent K_d value at least 4-fold higher was obtained for kaempferide at pH 9.0. Kaempferide was therefore used for further characterization, at pH 6.8, of the flavonoid binding site within H_6-NBD2.

Fig. 3 shows a total antagonism of binding to the domain between the nucleotide–analogue MANT-ATP and kaempferide. Indeed, the binding of MANT-ATP, as monitored by increase of extrinsic fluorescence (K_d = 21.0 ± 2.9 μM), was efficiently inhibited by preincubation with increasing concentrations of kaempferide, up to 10 μM (Fig. 3*A*). In addition, bound MANT-ATP, at nearly 0.5 mol/mol of H_6-NBD2 upon incuba-

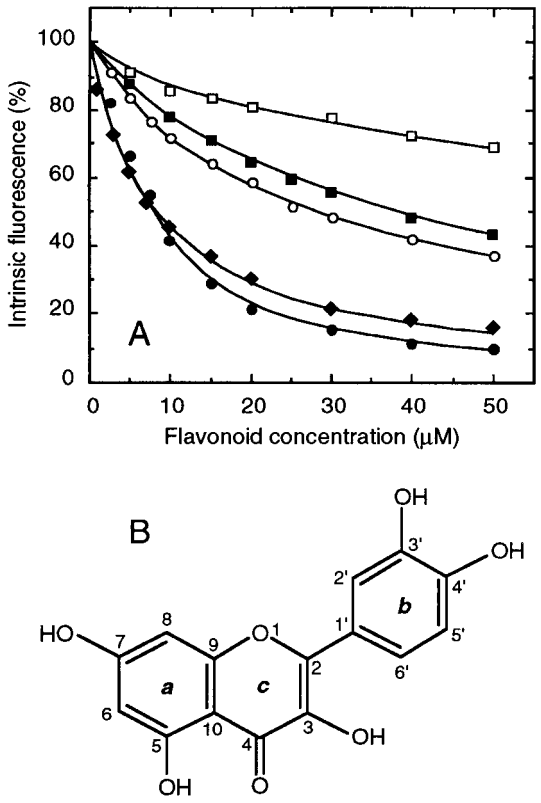

FIG. 2. Differential quenching of H_6-NBD2 intrinsic fluorescence by quercetin and other flavonoid modulators. (*A*) The interaction between different classes of flavonoids and 1.1 μM H_6-NBD2 was studied under the same conditions as described for MANT-ATP in Fig. 1*C*. Increasing concentrations of either quercetin [3,5,7,3′,4′-pentahydroxyflavone (●), whose chemical structure is shown in *B*], apigenin [5,7,4′-trihydroxyflavone (◆)], naringenin [2,3-dihydroapigenin (○)], genistein [isoapigenin (■)], or rutin [3-*O*-glucorhamnosyl quercetin (□)] were used as dimethyl sulfoxide solutions, up to a 2% (vol/vol) final concentration.

Table 1. Role of flavone substituents on the affinity for H_6-NBD2

Flavonoid	Substituents 3	5	7	4′	Apparent K_d, μM*
Flavone					34.1 ± 11.5
3-hydroxy flavone	OH				10.1 ± 2.5
7-hydroxy flavone			OH		34.9 ± 4.5
Chrysin		OH	OH		8.9 ± 0.3
Galangin	OH	OH	OH		5.3 ± 0.1
Kaempferol	OH	OH	OH	OH	6.7 ± 0.3
Kaempferide	OH	OH	OH	OCH_3	4.5 ± 0.2

*The H_6-NBD2 domain was incubated under conditions of Fig. 2 with increasing concentrations of substituted flavones; the quenching of intrinsic fluorescence was plotted as a function of concentration, and the apparent K_d was determined by using the GRAFIT program. For the different flavones, the average maximal quenching was 100.2 ± 5.9%.

tion with 20 μM MANT-ATP in the absence of kaempferide, was gradually displaced by the addition of increasing concentrations of kaempferide, inducing a nearly complete loss of extrinsic-fluorescence enhancement around 20 μM kaempferide (Fig. 3*A Inset*). The kaempferide-induced release of bound MANT-ATP could also be visualized by the gradual decrease in fluorescence resonance-energy transfer around 429 nm; a complete loss was obtained at 20 μM kaempferide, whereas the tryptophan residue still exhibited residual fluorescence (Fig. 3*B*).

In contrast, only a partial inhibition was produced by ATP against kaempferide binding (Fig. 4*A*). The concentration-dependent effect was maximal at 30 mM ATP and led to a 4-fold increase in the kaempferide concentration required to produce a 50% quenching of initial intrinsic fluorescence. Reciprocally, the 50% quenching produced by 4.5 μM kaempferide in the absence of ATP was partly released by addition of increasing concentrations of ATP up to 40 mM. The final residual fluorescence was

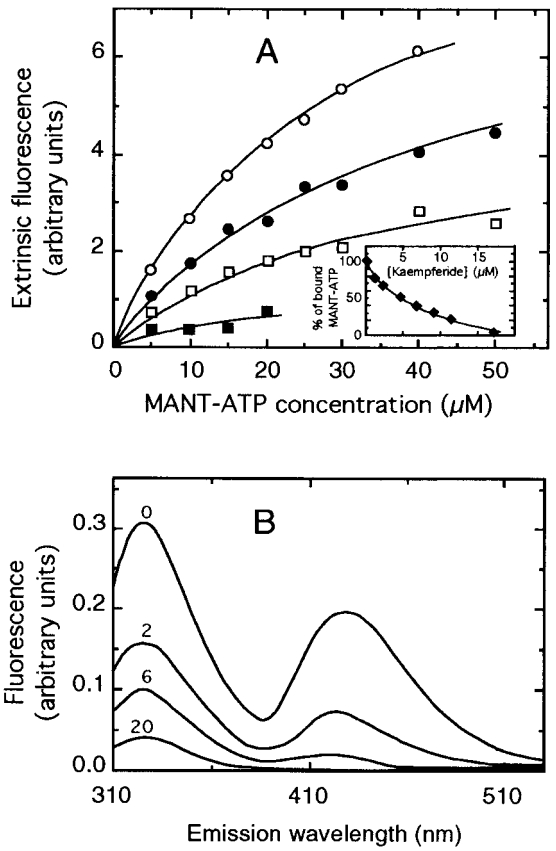

FIG. 3. Complete antagonism of kaempferide against MANT-ATP binding to H_6-NBD2. (*A*) The H_6-NBD2 domain (6 μM) was preincubated without (○) or with 3 (●), 6 (□), or 10 (■) μM kaempferide, and then assayed for MANT-ATP binding by the increase in extrinsic fluorescence between 420 and 460 nm upon excitation at 350 nm. (*Inset*) Decrease in extrinsic fluorescence related to displacement of bound MANT-ATP, upon incubation of 3.2 μM H_6-NBD2 with 20 μM MANT-ATP, by addition of increasing kaempferide concentrations. (*B*) The residual fluorescence resonance-energy transfer, produced upon incubation of 2.1 μM H_6-NBD2 with 20 μM MANT-ATP and excitation at 295 nm under conditions specified in Fig. 2*A*, was measured after addition of increasing kaempferide concentrations, from 0 to 20 μM, as indicated on each trace.

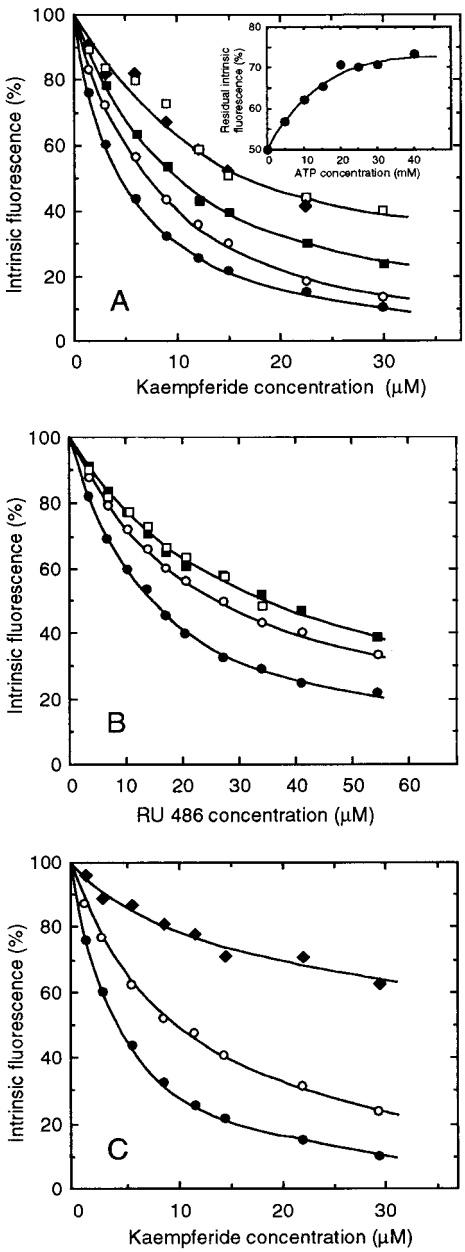

FIG. 4. Partly antagonistic bindings of RU 486, kaempferide, and ATP. (*A*) The H_6-NBD2 domain was preincubated at 1.1 μM in the absence (●) or presence of ATP at 10 (○), 20 (■), 30 (□), or 40 (◆) mM and assayed for quenching of intrinsic fluorescence upon addition of increasing kaempferide concentrations. (*Inset*) The 50% quenching produced by incubation of H_6-NBD2 with 4.5 μM kaempferide was partly released upon addition of increasing ATP concentrations, up to 40 mM. (*B*) Partial prevention against RU 486 binding (●) by preincubating H_6-NBD2 with 5 (○), 10 (■), or 20 (□) μM kaempferide. (*C*) Partial prevention against kaempferide binding (●) by preincubation with 24 μM RU 486 alone (○) and additional effect by preincubation with both 24 μM RU 486 and 20 mM ATP (◆).

approximately 75% of the initial value, indicating that bound kaempferide was only partly displaced (Fig. 4*A Inset*).

Fig. 4*B* shows that the H_6-NBD2 domain was also able to bind the antiprogestin RU 486, with a complete quenching of intrinsic fluorescence and a K_d of 16.2 ± 0.5 μM. Other hydrophobic steroids, like progesterone and Δ^6-progesterone, previously shown to bind to N-terminal NBD1 and whole P-glycoprotein (10), were also found to bind to H_6-NBD2 with similar affinities, and a lower interaction was observed with more polar steroids found to be transported by P-glycoprotein (not shown). Similarly, cytotoxic drugs that are known to be efficiently transported, such as vinblastine, taxol, or daunorubicin, exhibited a significant, but low-affinity, binding to H_6-NBD2. The binding of RU 486 was not significantly modified by preincubation in the presence of 11.3 mM ATP, indicating separate binding sites. In contrast, preincubation with kaempferide increased up to 3-fold the RU 486 concentration required for 50% quenching of intrinsic fluorescence; a nearly maximal, but partial, effect was produced by a 20 μM kaempferide concentration. Reciprocally, a partial prevention of kaempferide binding was produced by preincubation with 24 μM RU 486 (Fig. 4*C*). An additional prevention was produced by ATP, because a much lower kaempferide binding was observed upon preincubation with both 24 μM RU 486 and 20 mM ATP, as compared with RU 486 alone and ATP alone (Fig. 4*A*).

DISCUSSION

Overexpression and purification of hexahistidine-tagged NBD2 have shown that flavonoids are bifunctional modulators of P-glycoprotein, because their binding site partly overlaps vicinal and cytosolic binding sites for ATP and steroid modulators.

This paper describes the preparation and purification of a highly soluble NBD2 domain by (*i*) fusion with an N-terminal hexahistidine peptide, (*ii*) shortening of the C-terminal side, and (*iii*) use of a high ionic strength buffer containing HECAMEG and glycerol, as also successfully used with N-terminal NBD1 (22). Interestingly, H_6-NBD2 was much more soluble (at least 90 as compared with 15 μM) and was obtained with a much higher yield (15 as compared with 4 mg protein from a 3-liter culture) than H_6-NBD1. The hexahistidine-tagged NBD2 corresponding to segment 1044–1224 appeared much more stable and more soluble than the longer segment 1025–1276 previously purified, under different conditions, after thrombin cleavage of a GST fusion protein (20). Tryptophan-1106 exhibited a hydrophobic environment with a low wavelength for maximal emission, 328 nm, which was markedly red shifted (about 20 nm) upon denaturation with guanidine. The tryptophan residue was located in proximity to bound nucleotide as previously suggested from interaction with another ATP analogue, 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (20). In this work, MANT-ATP is shown to induce a high quenching of the domain intrinsic fluorescence, correlated to fluorescence resonance-energy transfer. The high efficiency ($E = 75\%$) reveals proximity between tryptophan-1106 and bound MANT-ATP. The marked blue shift in maximal extrinsic-fluorescence wavelength of bound MANT-ATP, from 444 to 429 nm, also indicates a hydrophobic environment around the MANT group.

All flavonoids reported to modulate drug efflux in MDR cancer cells, such as kaempferol, galangin, quercetin and hydrophobic derivatives, and genistein (11, 12, 28) are shown here to bind, with similar affinities and relative efficiencies, to purified cytosolic H_6-NBD2. In addition, rutin, a glycosylated flavonoid that did not behave as a modulator of cellular MDR phenotype (12), exhibits a very low-affinity binding for H_6-NBD2. Flavones, which are known as the best modulators, especially methoxylated derivatives, indeed display higher affinity for quenching H_6-NBD2 fluorescence as compared with isoflavones and flavanones. The same flavones were also found to bind to extended NBD1 domain by quenching the fluorescence of tryptophan-696; the apparent lower affinity observed in that case might be at least partly attributed to the relatively alkaline pH of 9.0, necessary to keep soluble the N-terminal domain (G.D., G.C., and A.D.P., unpublished data). The nearly complete maximal quenching of H_6-NBD2 intrinsic fluorescence produced by the most efficient flavonoids suggests that tryptophan-1106 would be located in very close proximity to the bound flavonoid. The antagonism of binding to H_6-NBD2 between kaempferide and ATP or MANT-ATP indicates that the flavonoid-binding site overlaps the nucleotide-binding one. This agrees with flavonoid inhibition of various ATP-binding proteins such as mitochondrial ATPase (29), myosin (30), Na^+/K^+ and Ca^{2+} plasma membrane ATPases (31, 32), protein kinase A (33), protein kinase C (34) and other serine/threonine protein kinases (35), tyrosine protein kinase (36), and topoisomerase II (37). The key role played here by a hydroxyl group at position 5 in the affinity for quenching H_6-NBD2 fluorescence is consistent with a structural homology of substituted rings *a* and *c* with the adenine moiety of ATP, based on the direct demonstration provided by the high-resolution structure of cyclin-dependent protein kinase 2 cocrystallized with a eight-substituted derivative of chrysin, L868276 (38). The 5-hydroxyl of L868276 is shown to interact with as many as five residues normally interacting with the 6-amino group of the adenine moiety of ATP, whereas the vicinal 4-carbonyl of L868276 appears equivalent to the 1-nitrogen atom of adenine. In contrast, the 7-hydroxyl does not seem to play any role, because it only binds a water molecule. The recent cocrystallization of quercetin with tyrosine kinase Hck exhibited a slightly different orientation, but confirmed the role of the 3-hydroxyl, in addition to the 4-carbonyl, for mimicking the adenine moiety of nucleotide (39). The 3,5-hydroxylated flavone, kaempferide, is indeed shown here to antagonize nucleotide binding when using either natural ATP or its high-affinity fluorescent derivative, MANT-ATP. Partial antagonism between flavonoids and ATP at the nucleotide binding-site of cytosolic NBD2 is consistent with the partly competitive inhibition produced by quercetin on the ATPase activity of whole P-glycoprotein (13) and its reverting effect on the MDR phenotype of MCF-7 cancer cells (12). Also, in the cystic fibrosis transmembrane conductance regulator, another ATP-binding cassette transporter, genistein, was recently suggested to interact at a nucleotide-binding site (40, 41).

The C-terminal cytosolic H_6-NBD2 domain is shown here to bind the antiprogestin RU 486, and other hydrophobic steroids, with similar features as the *N*-terminal cytosolic domain recently characterized (10). The latter domain was shown to contain a hydrophobic region, adjacent to the ATP-binding site, which was involved in steroid binding similar to the whole P-glycoprotein, and could also interact with the hydrophobic MANT group of

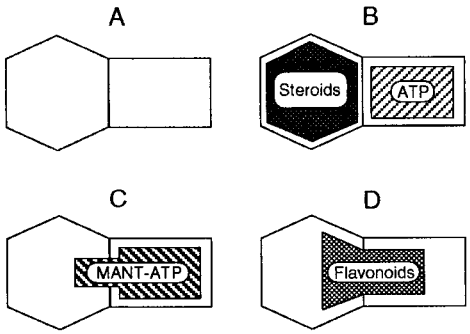

FIG. 5. Tentative model for binding of steroids, ATP, MANT-ATP, and flavonoids to adjacent nucleotide- and modulator-binding sites within the cytosolic H_6-NBD2 domain of P-glycoprotein. Previous work with purified cytosolic NBD1 domain (10) showed the existence of a steroid-binding region (hexagon) adjacent to the nucleotide site (rectangle), where RU 486 and ATP bind separately or together, and a mutually exclusive MANT-ATP binding, indicating partial overlapping of the vicinal steroid-binding region by the hydrophobic MANT group. These results indicate a similar behavior of cytosolic NBD2 (*A–C*), and additionally show that flavonoids are bifunctional modulators that partly overlap both ATP and steroid adjacent sites (*D*).

bound MANT-ATP (10) (Fig. 5*A–C*). The presence of two modulator-interacting regions, one on each cytosolic nucleotide-binding domain, is consistent with conclusions drawn from either affinity labeling (42), modifications of ATPase activity (43), anticancer drug efflux (44), or mutations (45, 46) of P-glycoprotein. It also agrees with the existence of two different drug-binding sites (47, 48), one of which is located on the cytosolic side of the transporter (49). The partial antagonism observed here between kaempferide and RU 486, and the higher antagonism of kaempferide against MANT-ATP as compared with ATP, for binding to H_6-NBD2, indicates that the flavonoid indeed binds to the steroid-interacting hydrophobic region. Such an interaction might play a critical role in the modulatory activity of flavonoids and would be additive to the partial structural homology with adenine inside the ATP site (Fig. 5*D*). This is consistent with the observation that quercetin was able to inhibit not only ATPase activity (13) but also Hoechst 33342 transport (50) by purified P-glycoprotein reconstituted into liposomes. Our results also agree with the significant inhibition produced by genistein on the photolabeling with azidopin (28). In addition, genistein inhibited daunorubicin transport by the MDR-associated protein, in a similar competitive way as other, common modulators such as cyclosporin A, PSC833, and verapamil (51). This work provides evidence for bifunctional modulators interacting at the cytosolic moiety of P-glycoprotein, as opposed to the main characterized modulators, which interact within the membrane moiety and are transported (5). The significant increase in affinity for quenching produced by hydrophobic substitution at position 4′ in kaempferide might indicate a possible interaction of ring *b* with the modulator-interacting hydrophobic region. Additional hydrophobic substitution at position 3′ increased further the binding affinity (unpublished data). It is worth mentioning that ring *b* in cocrystallized flavonoid derivative L868276 (38) or quercetin (39) interacted with protein sequences located outside the ATP-binding site.

In conclusion, flavonoids are shown here to constitute a new class of bifunctional modulators, which are able to partly overlap the ATP binding site, and a vicinal hydrophobic region interacting with steroids, within a cytosolic domain of P-glycoprotein. This important finding opens exciting perspectives for studying the molecular mechanism of interaction with flavonoid modulators through structural and functional approaches and for further design of a new generation of bifunctional and specific inhibitors of P-glycoprotein activity.

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