CMLS, Cell. Mol. Life Sci. 60 (2003) 2164–2177
1420-682X/03/102164-14
DOI 10.1007/s00018-003-3177-6

CMLS Cellular and Molecular Life Sciences

Research Article

Multiple flavonoid-binding sites within multidrug resistance protein MRP1

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Received 1 May 2003; received after revision 18 June 2003; accepted 24 June 2003

Abstract. Recombinant nucleotide-binding domains (NBDs) from human multidrug resistance protein MRP1 were overexpressed in bacteria and purified to measure their direct interaction with high-affinity flavonoids, and to evaluate a potential correlation with inhibition of MRP1-mediated transport activity and reversion of cellular multidrug resistance. Among different classes of flavonoids, dehydrosilybin exhibited the highest affinity for both NBDs, the binding to N-terminal NBD1 being prevented by ATP. Dehydrosilybin increased vanadate-induced 8-N_3-[α-^{32}P]ADP trapping, indicating stimulation of ATPase activity. In contrast, dehydrosilybin strongly inhibited leukotriene C_4 (LTC_4) transport by membrane vesicles from MRP1-transfected cells, independently of reduced glutathione, and chemosensitized cell growth to vincristine. Hydrophobic C-isoprenylation of dehydrosilybin increased the binding affinity for NBD1, but outsite the ATP site, lowered the increase in vanadate-induced 8-N_3-[α-^{32}P]ADP trapping, weakened inhibition of LTC_4 transport which became glutathione dependent, and induced some cross-resistance. The overall results indicate multiple binding sites for dehydrosilybin and its derivatives, on both cytosolic and transmembrane domains of MRP1.

Key words. Multidrug resistance protein 1 (MRP1); multidrug resistance (MDR); flavonoid; nucleotide-binding domain (NBD); chemosensitization; drug-binding site; ABC transporter.

Multidrug resistance protein 1 (MRP1) is an ATP-binding cassette (ABC) family transporter involved in cancer cell multidrug resistance (MDR phenotype) [1]. It transports a number of hydrophobic drugs, the conjugation of which with either glutathione (GSH), sulfate, or glucuronate [2] increases overall hydrophilicity. Neutral/basic amphipathic drugs, such as vincristine and daunorubicin [3, 4], as well as oxyanions, such as arsenite and antimonite [5], are cotransported with GSH. Leukotriene C_4 (LTC_4), an inflammatory mediator, is the endogenous GSH-conjugated substrate with highest affinity for MRP1 [6, 7]. MK571, developed as a cysteinyl-leukotriene antagonist, is considered to be a specific and potent MRP1 activity inhibitor [7, 8].

MRP1 belongs to the same C branch of ABC transporters as cystic fibrosis transmembrane conductance regulator (CFTR). Sequence alignments show that the N-terminal nucleotide-binding domain (NBD1) lacks a central 13-residue sequence between the Walker A and B motifs, as compared to the C-terminal nucleotide-binding domain (NBD2) and many NBDs from other ABC transporter branches. The two nucleotide-binding sites play different roles in MRP1 [9–11]: the site present on

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NBD1 is considered to be regulatory, exhibiting preferential binding of ATP over NBD2, and a low rate of hydrolysis; in contrast, the site present on NBD2 is catalytic, with a high rate of ATP hydrolysis, characterized by vanadate-induced trapping of ADP generated through hydrolysis. The two sites operate cooperatively, since ATP binding to NBD1 increases ATP hydrolysis and related vanadate-induced ADP trapping on NBD2 [12].

Fewer potential modulators have been reported for MRP1 by comparison to the other multidrug ABC transporter P-glycoprotein. Some recent examples are tricyclic isoxazoles [13] that bind in a GSH-dependent manner to the two last transmembrane segments [14]. Isoflavonoids such as genistein have been found to interact with MRP1 by stimulating ATPase activity [15, 16] and inhibiting transport activity at high concentrations [16–18] by competition with daunorubicin [19]. Other more active flavonoids, such as quercetin, have been recently discussed [20], but the binding site(s) within MRP1 remain unknown. Interestingly, genistein is also known to influence CFTR [21, 22], and direct inhibition of ATPase activity of purified recombinant NBD2 of CFTR has been reported [23]. Since flavonoids have been shown to bind to the cytosolic NBDs of both P-glycoprotein [24] and a homologous parasite transporter [25], with especially high affinity for isoprenylated derivatives with prenyl or geranyl substituents at position 6 or 8 [26, 27], we asked whether flavonoids might bind to the cytosolic domains of MRP1 and, if so, how this might affect the transport activity and the sensitivity of cell growth to anticancer drugs. To answer these questions, we needed to generate purified recombinant NBDs without fusion to either glutathione S-transferase, known to bind flavonoids [28, 29], or maltose-binding protein that might interact with glycosyl derivatives.

This work describes the overexpression in bacteria and purification of hexahistidine-tagged NBDs of human MRP1, as soluble and active proteins. A series of flavonoids indeed bind to both NBDs, with especially high affinity for the flavonolignan dehydrosilybin (DHS), whose binding overlaps the ATP site within NBD1. However, this cytosolic DHS-binding site is not responsible for inhibition of MRP1-mediated LTC_4 transport and related reversion of cellular MDR, implying additional membrane binding site(s). Isoprenylated derivatives of DHS behave differently, since they bind with higher affinity to NBDs, outside the ATP site, confer glutathione dependency to inhibition of LTC_4 transport, and induce cellular cross-resistance.

Materials and methods

Materials

The plasmid pT7-7/6His was a generous gift from J.-C. Cortay (Lyon, France). The oligonucleotide primers were purchased from MWG-Biotech Ebersberg, Germany. The mammalian expression vector pRC/RSV (Invitrogen, San Diego, Calif.) containing a 5508-bp MRP1 cDNA was a generous gift from M. Kool and P. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The M13K07 Helper Phage was from Life Technologies (Cergy-Pontoise, France). The *Escherichia coli* strain dut$^-$ ung$^-$ F′ CJ236 was from Bio-Rad (Marnes-la-Coquette, France). Restriction enzymes were purchased from either New England Biolabs (Beverly, Mass.) or Promega (Madison, Wis.), and Benzonase was from Merck (Darmstadt, Germany). The Ni^{2+}-nitrilotriacetic acid agarose gel was from Qiagen (Courtaboeuf, France), and TNP-ATP from Molecular Probes (Eugene, Ore.). The PWO DNA polymerase and ATP came from Roche Molecular Biochemicals (Meylan, France), HECAMEG [6-O-(N-heptylcarbamoyl)-methyl-α-D-galactopyranoside] from Interchim (Montluçon, France) and IPTG (isopropyl-1-thio-β-D-galactopyranoside) from Euromedex (Mundolsheim, France). Protein molecular-weight markers for SDS-polyacrylamide gel electrophoresis (low-molecular-weight markers, range 14.4–97 kDa) and for Western blot (Prestained SDS-PAGE standards, broad range, 6.9–210 kDa) were from Amersham Biosciences (Courtaboeuf, France) and Bio-Rad respectively. Mouse monoclonal antibody MRPm6 was purchased from Alexis Biochemicals (Illkirch, France) and mouse monoclonal antibody Anti-His_6 from Roche Molecular Biochemicals. Goat anti-mouse-AP conjugate and the AP conjugate substrate kit for Western blot development were from Bio-Rad. Genistein, apigenin and quercetin (HPLC grade) were from Extrasynthèse (Genay, France); naringenin and silybin were from Sigma (Saint Quentin-Fallavier, France), and galangin from Aldrich (Saint Quentin-Fallavier, France). DHS and its isoprenylated derivatives, whose structures are shown on fig. 4A, were synthesized as described previously [30]. Imidazole, GSH, sodium orthovanadate, and vincristine were from Sigma. [14, 15, 19, 20-^{3}H]LTC_4 was purchased from NEN Life Science Products (Boston, Mass.) and 8-N_3-[α-^{32}P]ATP from Affinity Labeling Technologies (Lexington, Ken.). Tissue culture materials were from Life Technologies (Cergy-Pontoise, France).

Construction of expression vectors

The pRC/RSV vector containing the MRP1 coding sequence was used as a template for PCR preparation of the DNA sequences 1882–2643 and 3865–4593, coding for NBD1 (628–881 amino acid sequence) and NBD2 (1289–1531 amino acid sequence, respectively). The forward and reverse primers provided by MWG-Biotech were 5′-TAT AAT TGG ATC CGA CAG CAT CGA GCG ACG GCC TGT CAA AGA CGG CGG GGG CAC-3′ and 5′-TAT AAT TAA GCT TCA GTT CTC CTC TGC ATC CTG CTC CTG CTC TGT GCT GGC ATA-3′ for

NBD1, and 5′-GGA TTC CAG GTG GGC CGA GTG GAA TTC CGG-3′ and 5′-AAG CTT CAC ACC AAG CCG GCG TCT TTG GC-3′ for NBD2. The primers allowed the introduction of *Bam*HI and *Hin*dIII restriction sites. The PCR product containing the NBD2 coding sequence was first integrated into the plasmid pCAP, using the PCR Cloning Kit (blunt end) from Roche Molecular Biochemicals, before digestion with *Bam*HI and *Hin*dIII. The PCR product containing the NBD1 coding sequence was purified and digested with the two enzymes. The products were ligated into the corresponding sites of linearized pT7-7 plasmid including an N-terminal hexahistidine coding sequence [31] and *E. coli* BL21(DE3) cells were transformed with the ligation product. The expression vector containing the NBD1 coding sequence was used as a template for site-directed mutagenesis to reverse a preexisting mutation C(2333)A leading to a lysine at position 713 of the protein instead of a glutamine, which is highly conserved among many wild-type ABC transporters. This mutation was present in the entire MRP1 cDNA due to a PCR error. A-to-C reversion at position 2274 was achieved by oligonucleotide-directed mutagenesis with the Kunkel method [32, 33]. The NBD1 coding sequence was subcloned in the phagemid pBluescript IIKS(+) (Stratagene, Amsterdam, The Netherlands). The uracil-containing DNA template was prepared in *E. coli* CJ236 cells [F′[camr(pCJ105)] dut-1, ung-1, thi-1, relA1] transformed with the phagemid and infected with the M13K07 Helper Phage. The oligonucleotide 5′-TCC AGG CCT GCT GGG GCA CAT AGG CC-3′ contains the mutation and suppresses the single restriction site for *Xcm*I initially present in the NBD1 coding sequence. The oligonucleotide was phosphorylated with T4 polynucleotide kinase, annealed to the single-stranded circular template, and then treated with T4 DNA polymerase and T4 DNA ligase to produce a double-stranded circular molecule. After transformation of *E. coli* JM109 with this DNA, the wild-type coding sequence was recovered upon digestion of the pBluescript with *Bam*HI and *Hin*dIII, and recloned in the pT7-7/6His plasmid. All constructs were verified by DNA sequencing (Genome Express, Grenoble, France).

Overexpression and purification of recombinant proteins

The purified plasmid was introduced into competent *E. coli* strain BL21(λDE3) and grown overnight on Luria Broth containing 100 µg/ml ampicillin. Expression of the recombinant proteins was induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) when the absorbance at 600 nm reached 0.7. The induction was performed at 25 °C during 4 h for NBD1 or 2 h for NBD2. The cells were harvested by centrifugation at 5500 g for 10 min at 4 °C, and resuspended in 10 mM Tris-HCl, pH 8.5, 250 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 7 mM $MgCl_2$, 2% lauryldimethylamine oxide, 2 mM phenylmethylsulfonyl fluoride, 5 µM leupeptine, 5 µM pepstatin, and 25 units Benzonase/ml. The cells were lysed twice using a SLM-Aminco French Pressure Cell press at 1000 psi with a 40-ml cell, and centrifuged at 30,000 g for 25 min. The supernatant was first diluted twofold in a buffer containing 40 mM potassium phosphate, pH 8.5, 20% glycerol, 1 M NaCl, 50 mM imidazole, 10 mM β-mercaptoethanol, and then applied to a Ni^{2+}-nitrilotriacetic acid column equilibrated with 20 mM potassium phosphate, pH 8.5, 1 M NaCl, 10% glycerol, 1% lauryldimethylamine oxide, 25 mM imidazole, 5 mM β-mercaptoethanol. The column was first extensively washed with the same buffer and then with the same buffer containing only 0.3% lauryldimethylamine oxide and 0.5 M NaCl, and finally with the latter buffer in the absence of lauryldimethylamine oxide but in the presence of 0.01% HECAMEG. Contaminants were preeluted with 50 mM imidazole (in the presence of 20 mM potassium phosphate, pH 8.5, 20% glycerol, 0.5 M NaCl, 0.01% HECAMEG, 5 mM β-mercaptoethanol); the retained proteins were then eluted with 250 mM imidazole (in the presence of 20 mM potassium phosphate, pH 8.5, 20% glycerol, 0.5 M NaCl, 0.01% HECAMEG, 5 mM β-mercaptoethanol). The fractions were pooled and dialysed against either 30 mM Tris-HCl, pH 7.2, 20% glycerol, 5 mM β-mercaptoethanol, 50 mM NaCl, 0.05% HECAMEG for studying the interactions of NBDs with TNP-ATP, or 30 mM Tris-HCl, pH 7.2, 20% glycerol, 5 mM β-mercaptoethanol, 500 mM NaCl, 0.01% HECAMEG for flavonoid interactions. The dialysate was centrifuged to discard possible traces of precipitated material; the supernatant was aliquoted and kept frozen in liquid nitrogen. Protein fractions were analyzed on SDS-polyacrylamide gel electrophoresis [34]. Protein concentration was routinely determined by the method of Bradford [35] with the Coomassie Plus Protein Assay Reagent Kit from Pierce (Brebières, France).

Interactions of purified NBDs with nucleotides and flavonoids

The binding of TNP-ATP, monitored by enhancement of extrinsic fluorescence, and displacement experiments upon ATP addition were performed as previously described for P-glycoprotein NBDs [36, 37]. Interaction with flavonoids was measured by quenching of protein intrinsic fluorescence, after correction for the inner-filter effect with N-acetyltryptophanamide, as detailed for NBD2 of P-glycoprotein [24] or parasite homologue [25]. Data analysis and dissociation constant determination were performed with the Grafit program (Erithacus software). The chase of bound TNP-ATP by increasing ATP concentrations was analyzed by the equation of Stinson and Holbrook [38] assuming competitive binding. ATPase activity was assayed by a spectrophotometric

method using an ATP-regenerating system as previously described [36].

Membrane vesicle preparation, and assays of vanadate-induced nucleotide trapping and LTC_4 transport

Stably MRP1-transfected baby hamster kidney (BHK-21) cells [39] were disrupted at 800 psi in a Parr N_2 cavitation bomb, and the membranes were collected by differential centrifugation, suspended in 10 mM Tris-HCl, pH 7.5, containing 250 mM sucrose and protease inhibitors (2 µg/ml aprotinin, 121 µg/ml benzamidine, 3.5 µg/ml E64, 1 µg/ml leupeptin, and 50 µg/ml Pefabloc), and passed through a Liposofast vesicle extruder as previously described [10]. Photoaffinity labeling of MRP1 proteins was carried out at 37 °C in 40 mM Tris-HCl, pH 7.5, 2 mM ouabain, 0.1 mM EGTA, 10 mM $MgCl_2$, 5 µM 8-N_3-[α-^{32}P]ATP (1 µCi), 800 µM vanadate, and flavonoids or MK571 from dimethylsulfoxide solutions (10% final concentration); the membanes were pelleted at 4 °C, washed by centrifugation and irradiated at 254 nm with a Stratalinker UV Crosslinker. The labeled proteins were separated on a 7%-polyacrylamide gel and counted with a Packard Instant Imager, as detailed elsewhere [10]. ATP-dependent transport of [^{3}H]leukotriene C_4 into the membrane vesicles was studied by incubating 3 µg of membrane proteins for 6 min at 37 °C in 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 10 mM $MgCl_2$, 200 nM [^{3}H]LTC_4 (17.54 nCi), 4 mM ATP and flavonoids from dimethylsulfoxide solutions (5% final concentration); the reaction was stopped by high dilution and rapid filtration through a nitrocellulose membrane which was counted by liquid scintillation, as described [10]. Within each experiment, determinations were carried out in triplicate.

Cell culture, flavonoid cytotoxicity and chemosensitization to vincristine

For cytotoxicity experiments [39], MRP1-transfected BHK-21 cells were cultured at 37 °C in 96-well plates, for 4 days in the presence of increasing concentrations of flavonoid (or MK571). MTT was added for an additional period of 4 h to determine residual living cells by absorbance at 570 nm. The IC_{50} value corresponded to the flavonoid (or MK571) concentration producing 50% cell death. For chemosensitization experiments, MRP1-transfected cells were cultured in the presence of increasing vincristine concentrations, in the absence or presence of flavonoid (or MK571) at a sub-cytotoxic concentration corresponding to 10–15% cell death, as determined from the above cytotoxicity experiments. The IC_{50} values corresponding to vincristine concentrations producing 50% cell death were determined under each culture condition. Two types of control cell, highly sensitive to vincristine, were used: parental, untransfected, BHK-21 cells on the one hand, and CFTR-transfected BHK-21 cells [40] on the other. A high level of MRP1 expression has been shown to characterize the MRP1-transfected cells by using the MRPr1 monoclonal antibody and detection by either immunochemical staining of whole cells or Western blot of cell lysate proteins, whereas the transporter was undetectable in both types of control cell [39]. For all MTT experiments, determinations were carried out in triplicate. For both cytotoxicity and chemosensitization experiments, the final dimethylsulfoxide concentration, as flavonoid solvent, was 0.5%; data analysis and IC_{50} determination were performed with the Grafit program (Erithacus software).

Results

Overexpression and purification of hexahistidine-tagged NBDs of human MRP1

NBDs of similar size, 254 residues for NBD1 and 243 residues for NBD2, were overexpressed with N-terminal hexahistidine tags. Figure 1A shows that NBD1, with an apparent molecular weight close to the 30-kDa marker, was highly induced by IPTG and represented the main protein band; it could be easily purified (> 95%) by single-step nickel-affinity chromatography. NBD2, with a similar apparent molecular weight, was overexpressed to a lesser extent, but could also be efficiently purified by nickel-affinity chromatography. Identification of the two NBDs was achieved by Western blots using either anti-hexahistidine monoclonal antibody for NBD1 or the specific MRPm6 monoclonal antibody for NBD2: in both cases, a single band was revealed which migrated around the 29-kDa prestained marker, indicating the absence of apparent degradation of the proteins (fig. 1B). Protein titration indicated that purified NBD1 was obtained with a fivefold higher yield than NBD2, i.e., 15–18 mg as compared to 3–4 mg per liter of bacterial culture.

Although a very low ATPase activity was observed for purified NBD1 (0–1 nmol/min × mg) and NBD2 (3–5 nmol/min × mg), both NBDs bound the ATP fluorescent analogue TNP-ATP with high affinity. Dissociation constant values of 1.95 ± 0.36 and 0.84 ± 0.14 µM for NBD1 and NBD2, respectively, were obtained (fig. 2). Bound TNP-ATP was efficiently displaced by addition of increasing concentrations of ATP, which most likely bound to the same nucleotide-binding site, with K_i values of 0.66 and 0.67 mM for NBD1 and NBD2, respectively, (fig. 2, insets).

Interactions of purified NBDs with different classes of flavonoids and isoprenylated derivatives

Recombinant NBDs, which contained one (NBD2) or two (NBD1) trypthophan residue(s), exhibited a characteristic intrinsic emission-fluorescence spectrum with a

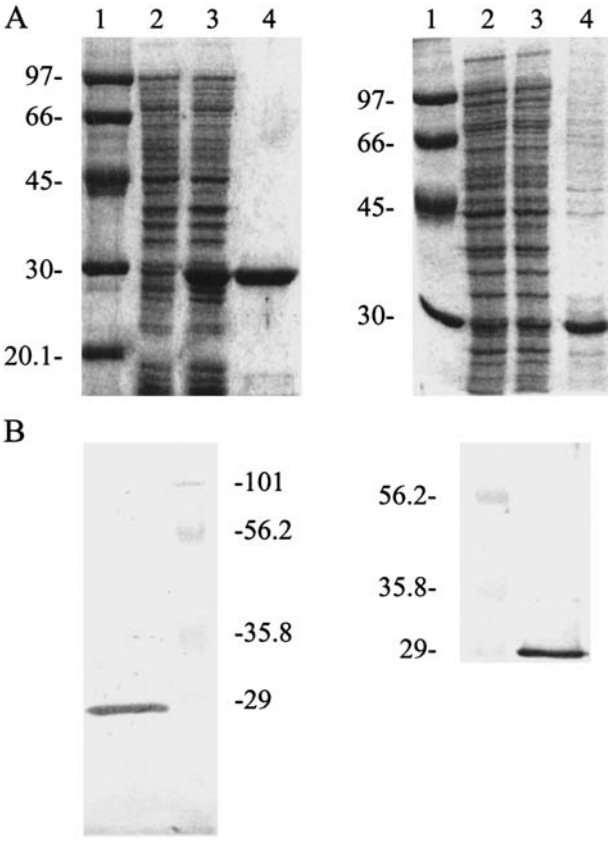

Figure 1. Overexpression and purification of MRP1 NBDs. (*A*) Fractions obtained from a culture of *E. coli* overexpressing the cDNA encoding either NBD1 (left) or NBD2 (right) of MRP1, as analyzed by polyacrylamide gel electrophoresis under denaturing conditions. Lane 1, molecular-weight markers as indicated in kDa; lane 2; total bacterial proteins before induction; lane 3; total bacterial proteins after induction; lane 4, purified domain eluted from nickel-chelate affinity chromatography. (*B*) Immunodetection by specific monoclonal antibodies. NBD1 was detected with the anti-hexahistidine antibody, and NBD2 with the MRPm6 specific antibody.

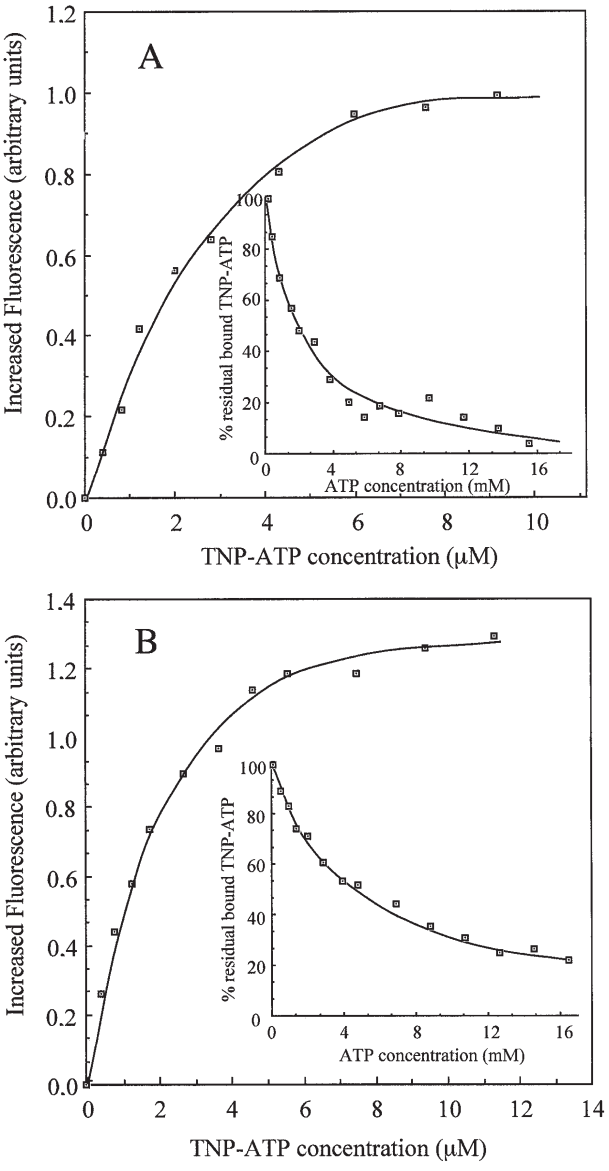

Figure 2. Nucleotide binding to purified NBDs. The increase in fluorescence of TNP-ATP upon binding to either NBD1 (*A*) or NBD2 (*B*), at 2 μM, was measured as a function of TNP-ATP concentration, and data were analyzed with the Grafit program. The chase of TNP-ATP bound to either 4 μM NBD1 (inset, *A*) or 5 μM NBD2 (inset, *B*) was studied by measuring the residual fluorescence upon addition of increasing ATP concentrations, as indicated.

maximal wavelength of 334–335 nm at neutral pH. A marked quenching of fluorescence was observed upon addition of various flavonoids (fig. 3). The efficiencies of different classes of flavonoid were compared using compounds containing three hydroxyl groups: at positions 5 and 7 of ring A in all cases, and either 4′ of ring B for genistein, naringenin, and apigenin, or 3 of ring C for galangin. For binding to NBD1 (fig. 3A), the isoflavone genistein, with ring B branched to ring C at position 3 instead of 2, exhibited the lowest affinity interaction (K_D > 100 μM), followed by the flavone apigenin (K_D = 47.9 ± 5.3 μM), the flavanone naringenin with a saturated 2,3-bond (K_D = 18.8 ± 5.1 μM), and the flavonol galangin (K_D = 17.9 ± 3.8 μM), as illustrated in table 1. Substitution of positions 3′ and 4′ of flavonols by hydroxyl groups in quercetin had no significant effect (K_D = 16.1 ± 2.5 μM), whereas a very strong increase in affinity was produced by the monolignol unit in DHS (K_D = 1.6 ± 0.18 μM). Reduction of the DHS 2,3-double bond in the flavanonol silybin markedly reduced the binding affinity (K_D = 22.2 ± 4.6 μM), the effect being higher than for the flavanone naringenin compared to the flavone apigenin. NBD2 (fig. 3B) behaved similarly to NBD1 for the most part, since comparable K_D values were obtained for genistein (84 ± 21 μM), apigenin (35.8 ± 4.5 μM), galangin (22.1 ± 4.6 μM), quercetin (12.3 ± 1.8 μM), and DHS (2.1 ± 0.24 μM). However, the flavanone naringenin (K_D = 62 ± 19 μM) and, to a lesser extent, the flavanonol sily-

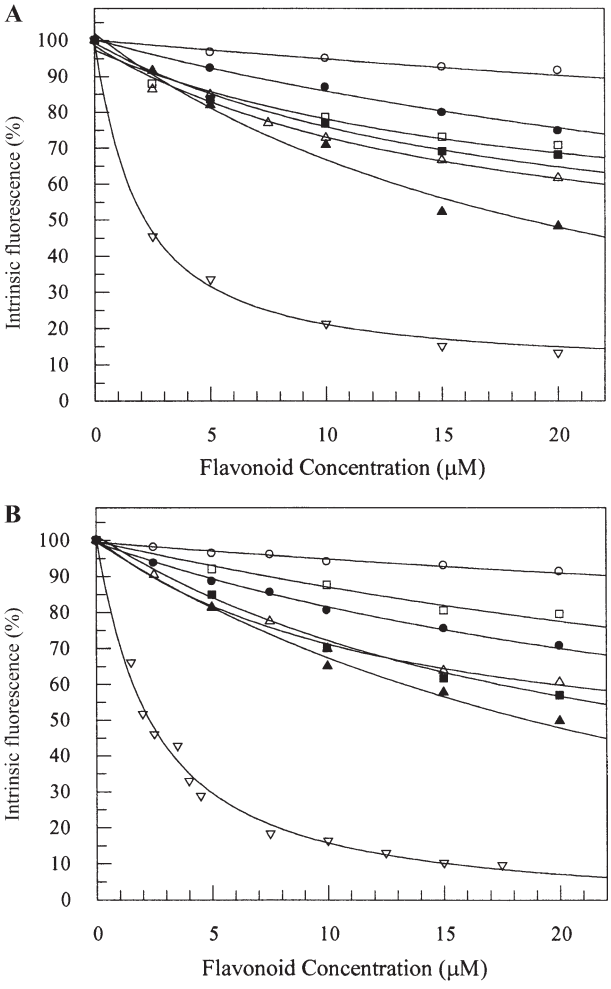

Figure 3. Binding of different classes of flavonoids to purified NBDs. The intrinsic fluorescence of purified NBD1 (*A*) or NBD2 (*B*) at 0.5 μM was measured in the presence of increasing concentrations of flavonoids belonging to different classes: genistein (open circle), naringenin (open square), apigenin (closed circle), galangin (closed square), quercetin (open triangle), silybin (closed triangle) or DHS (open inverted triangle).

bin (K_D = 30.4 ± 7.9 μM) bound with a lower affinity compared to NBD1. Since DHS exhibited the highest binding affinity for both NBDs, and 6- or 8-isoprenylation was previously found to further increase the affinity of binding to the parasite multidrug transporter [27], the prenyl and geranyl derivatives of DHS, whose structures are shown in figure 4A, were checked for interaction with MRP1 NBDs. Interestingly, these hydrophobic compounds bound preferentially to NBD1 compared to NBD2. Indeed, the binding affinities toward NBD1 (fig. 4B) were markedly increased for both prenylated (K_D = 0.16–0.27 μM) and geranylated (K_D = 0.12–0.16 μM) derivatives. In contrast, the binding affinity toward NBD2 (fig. 4C) was only increased for the more hydrophobic geranylated derivatives (K_D = 0.28–0.47 μM), whereas the K_D values for the less hydrophobic, prenylated, derivatives (1.2–2.8 μM) were not very different from that of DHS.

Table 1. K_D values for direct interaction of flavonoids and isoprenylated derivatives with MRP1 NBDs.

Flavonoid	K_D for NBD1 (μM)	K_D for NBD2 (μM)
Genistein	> 100	84 ± 21
Apigenin	47.9 ± 5.3	35.8 ± 4.5
Naringenin	18.8 ± 5.1	62 ± 19
Galangin	17.9 ± 3.8	22.1 ± 4.6
Quercetin	16.1 ± 2.5	12.3 ± 1.8
DHS	1.6 ± 0.18	2.1 ± 0.24
Silybin	22.2 ± 4.6	30.4 ± 7.9
6-Prenyl DHS	0.27 ± 0.03	2.8 ± 0.64
8-Prenyl DHS	0.16 ± 0.03	1.2 ± 0.50
6-Geranyl DHS	0.16 ± 0.01	0.47 ± 0.04
8-Geranyl DHS	0.12 ± 0.04	0.28 ± 0.03

The different flavonoids were incubated under conditions of figs 3 and 4, with either NBD1 or NBD2. The K_D values of direct binding were obtained by the Grafit program analysis of the quenching of intrinsic fluorescence as a function of flavonoid concentration.

The binding of unsubstituted DHS was antagonized by ATP, as illustrated in figure 5A. Preincubation with increasing ATP concentrations efficiently lowered the flavonolignan binding, either markedly to NBD1 (left) or moderately to NBD2 (right), with apparent K_i values of 1.2–1.7 mM and 9.4 mM, respectively (fig. 5A). In contrast, the higher-affinity binding of 8-geranyl-DHS was not significantly altered by preincubation of NBD1 with ATP (fig. 5B, left), and only minimally with NBD2, with a high apparent K_i around 30 mM (fig. 5B, right). All the other isoprenylated derivatives of DHS also exhibited no, or only limited, effects (at least 15-fold lower than that produced by DHS).

Effects of flavonoids on MRP1-mediated vanadate-induced ADP trapping and transport activity, in inverted vesicles of membranes from MRP1-transfected BHK-21 cells

Upon hydrolysis of 8-N_3-[α-^{32}P]ATP by MRP1 in the presence of magnesium ions and photoirradiation, a vanadate-induced retention of ADP derivative is observed, indicative of ATP hydrolysis mainly occurring at NBD2 [9–11]. The amount of trapped ADP was gradually increased upon addition of increasing flavonoid concentrations, with a nearly maximal effect at 25 μM. A large increase of 122% of vanadate-induced nucleotide trapping was produced by DHS (fig. 6A), whereas smaller effects were observed with isoprenylated derivatives (23–89% increase).

The flavonoid effects on MRP1 transport activity were assayed using [^{3}H]-LTC_4 (fig. 6B). A high-affinity inhibition was produced by DHS, with an IC_{50} value of 1.1 ± 0.25 μM, corresponding to an equal efficiency as the

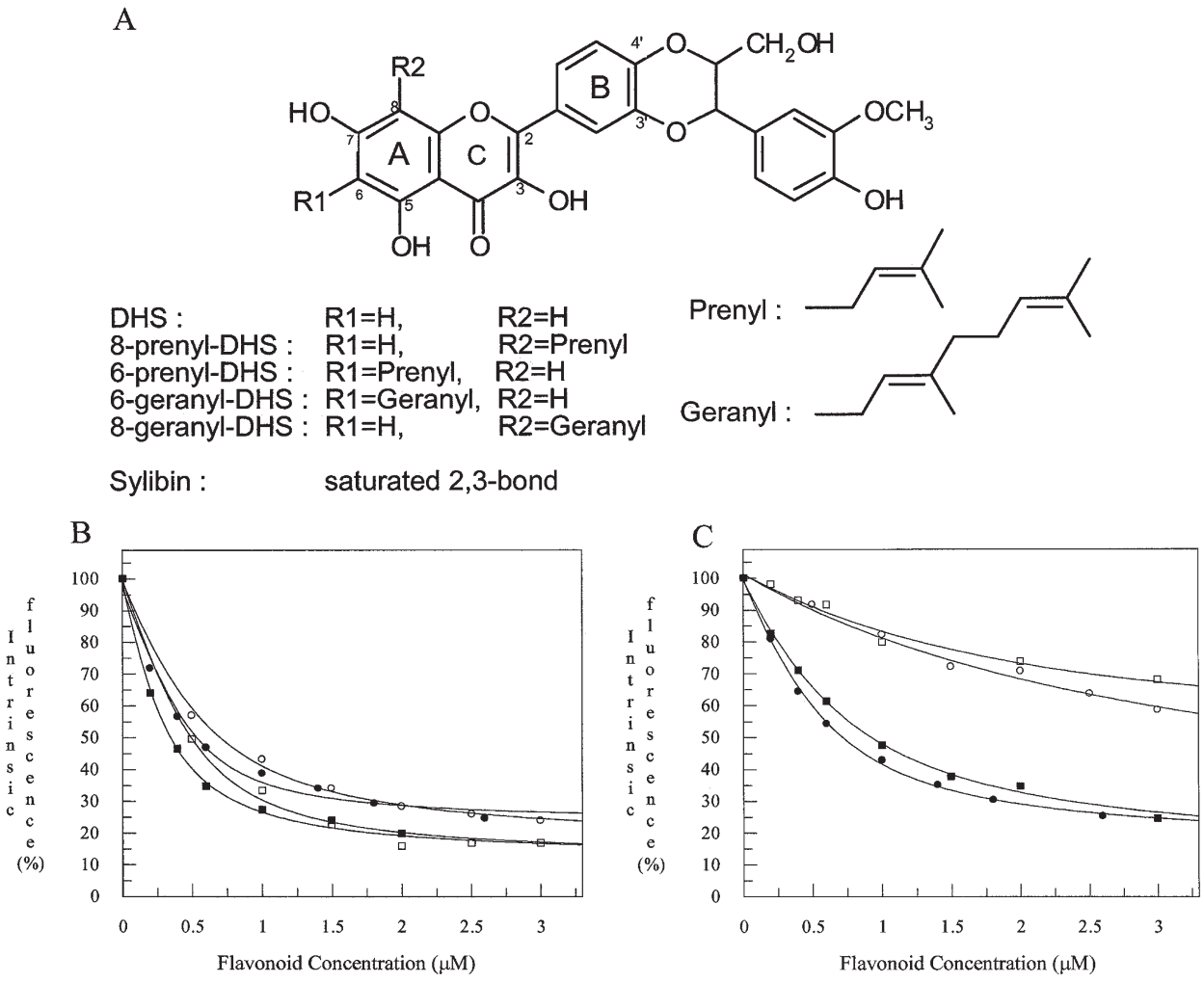

Figure 4. Structure of DHS isoprenylated derivatives, and binding to purified NBDs. Structure of silybin, DHS, and prenylated or geranylated derivatives used in this work (*A*): DHS was obtained from silybin by oxidation of the 2,3-bond, and a prenylated or geranylated substituent can be chemically introduced at either position 6 (R1) or 8 (R2). Binding of isoprenylated derivatives of DHS [6-prenyl-DHS (open circle), 8-prenyl-DHS (open square), 6-geranyl-DHS (closed square) or 8-geranyl-DHS (closed circle)] to NBD1 (*B*) or NBD2 (*C*) was measured at either 0.25 or 0.5 μM of NBDs, depending on the affinity.

MRP1-specific inhibitor MK571 (1.1 ± 0.20 μM). In contrast, all isoprenylated derivatives were much less efficient, with IC_{50} values of 3.9 ± 0.23, 7.7 ± 0.86, 6.8 ± 0.73, and 15 ± 1.2 μM for 8-prenyl, 8-geranyl, 6-prenyl and 6-geranyl derivatives, respectively. A low potency was also observed with the flavanonol silybin (IC_{50} = 16 ± 1.2 μM; fig. 6B). Interestingly, the addition of reduced glutathione diminished the differences in the inhibitory effects of these derivatives (fig. 6C). The potency of MK571 was insensitive to glutathione (1.1 ± 0.23 μM), and that of DHS was slightly lowered (1.3 ± 0.34 μM), in contrast to that of all other compounds which was greatly increased: IC_{50} values of 4.7 ± 0.40, 6.3 ± 0.44, and 4.1 ± 0.11 μM were obtained for silybin, 6-geranyl-DHS, and 8-geranyl-DHS, respectively, whereas potent effects (IC_{50} = 1.6-1.7 ± 0.11 μM), comparable to that of MK571 and DHS, were produced by the two prenylated derivatives. The inhibition by 8-prenyl-silybin was also 2.7-fold favored by glutathione (not shown here).

Intrinsic cytotoxicity of flavonoids to control and MRP1-transfected cells, and modulation of resistance to vincristine

DHS exhibited a rather low intrinsic cytotoxicity, with similar IC_{50} values in control and MRP1-transfected BHK-21 cells (14.6 ± 0.55 and 15.9 ± 0.46 μM, respectively) as shown in figure 7A. Isoprenylation increased flavonoid cytotoxicity in control cells: IC_{50} values of 12 ± 0.25, 8.9 ± 0.11, 6.6 ± 0.14, and 5.2 ± 0.10 μM were obtained with 6-prenyl-, 6-geranyl-, 8-prenyl-, and 8-geranyl derivatives of DHS, respectively (table 2). Interestingly, the increase in cytotoxicity was lower in MRP1-transfected compared to control cells; this indicated that

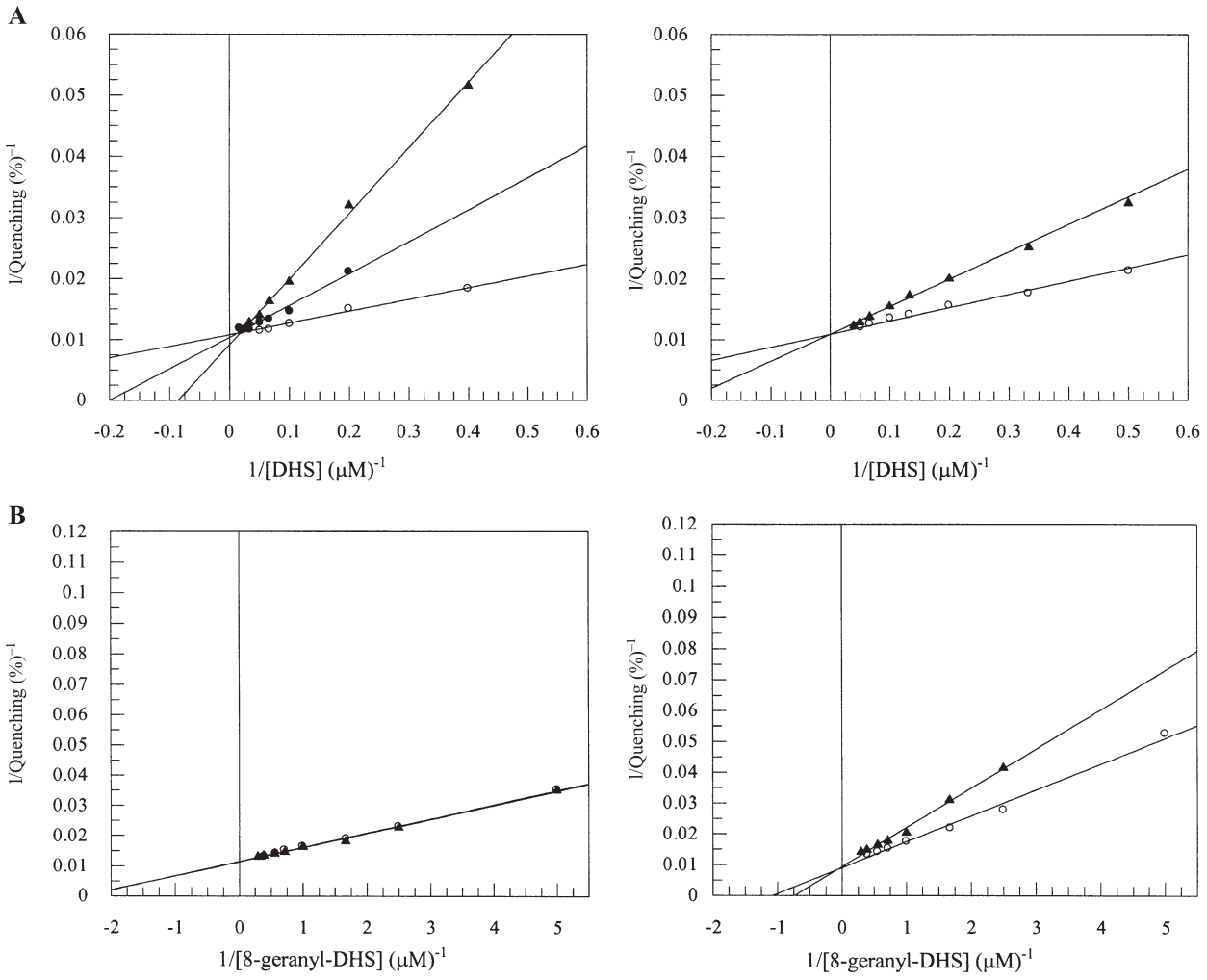

Figure 5. Interdependence between nucleotides and DHS derivatives. (*A*) Double-reciprocal plot of ATP prevention of the binding of DHS to either NBD1 (left) or NBD2 (right): ATP at either 0 (open circle), 2 (closed circle) or 10 (closed triangle) mM was preincubated with the domain for 30 min before addition of increasing flavonoid concentrations under the conditions of figure 4B, C. (*B*) The same experiments were performed with 8-geranyl-DHS, with ATP at either 0 (open circle) or 10 (closed triangle) mM.

MRP1 conferred some resistance to the isoprenylated compounds, with a resistance factor of up to 2.1 for 8-geranyl-DHS, and suggested that these compounds might be transported. The 8-prenyl derivative of silybin also produced cellular cross-resistance, with a 2.6 resistance index (not shown here).

When cells were grown in the presence of vincristine, sub-toxic concentrations of DHS were able to strongly lower resistance of MRP1-transfected cells, while a limited effect was produced on control cells (fig. 7B). In contrast, none of the prenylated derivatives was able to produce any differential effect between MRP1-transfected and control cells (table 3). No significant effect was either observed with silybin (not shown here). On the contrary, a qualitatively-similar, although quantitatively-smaller, decrease in resistance as with DHS was observed with the specific MRP1-inhibitor MK571.

Table 2. Intrinsic cytotoxicity of DHS and derivatives.

Flavonoid	IC_{50} (μM)	
	MRP1-transfected cells	control cells
DHS	15.9 ± 0.46	14.6 ± 0.55
6-Prenyl-DHS	15.1 ± 0.62	12.0 ± 0.25
6-Geranyl-DHS	8.6 ± 0.58	8.9 ± 0.11
8-Prenyl-DHS	9.3 ± 0.3	6.6 ± 0.14
8-Geranyl-DHS	10.8 ± 0.25	5.2 ± 0.10
Silybin	29.0 ± 0.9	31.5 ± 2.0

Cell growth experiments with the different DHS derivatives were performed under conditions of figure 7A. IC_{50} values were determined for MRP1-transfected cells and control cells, as illustrated in figure 7A.

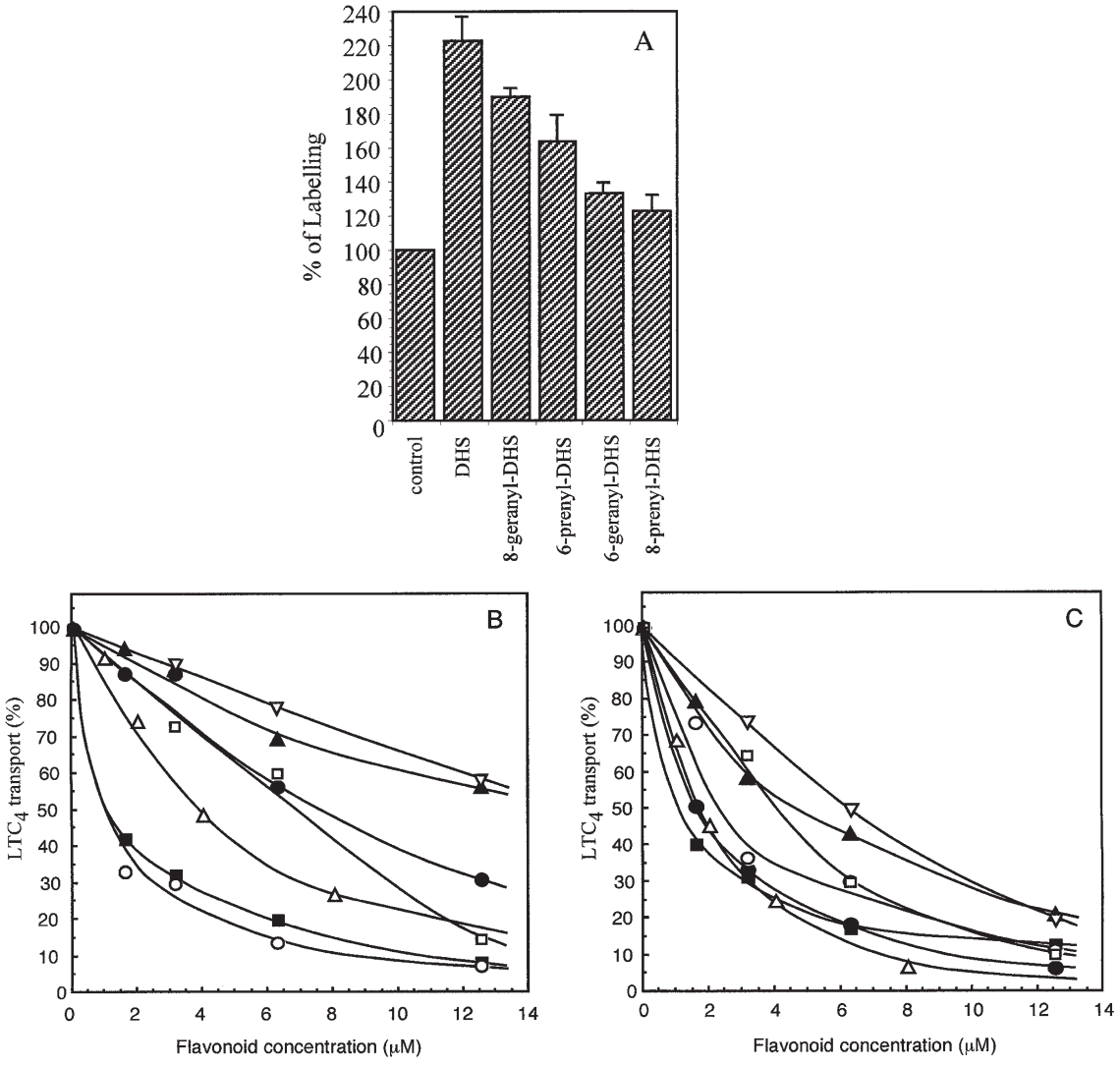

Figure 6. Stimulation by DHS and derivatives of vanadate-induced nucleotide trapping and inhibition of LTC_4 transport by MRP1-containing membrane vesicles. (*A*) Increase by 25 μM DHS or isoprenylated derivatives of vanadate-induced nucleotide trapping within membrane vesicles from MRP1-transfected cells: the latter were incubated with 8-N_3-[α-^{32}P]ATP in the presence of $MgCl_2$ and vanadate, photoirradiated, and assayed for retained radioactivity, as described in Materials and methods. The error bars corresponding to standard deviation are indicated. (*B, C*) The membrane vesicles prepared from MRP1-transfected cells were incubated for 6 min at 37 °C, in the absence (*B*) or presence (*C*) of 2 mM GSH, with 200 nM [^{3}H]LTC_4, 10 mM $MgCl_2$, and 4 mM ATP, together with increasing concentrations of either DHS (open circle), 8-prenyl-DHS (open triangle), 6-prenyl-DHS (closed circle), 8-geranyl-DHS (open square) or 6-geranyl-DHS (open inverted triangle). The radioactivity retained by vesicles was determined after rapid filtration, and corrected for background of controls conducted with AMP instead of ATP. For comparison, similar experiments were performed with silybin (closed triangle) or MK571 (closed square).

Discussion

Preparation of hexahistidine-tagged NBDs as active proteins

This work is the first to describe overexpression and purification of hexahistidine-tagged NBDs from MRP1 in high yields and as stable proteins. As much as 15–18 mg protein for NBD1 and 3–4 mg protein for NBD2 were obtained from one liter of culture, similar to yields for fusions with either glutathione S-transferase or maltose-binding protein [41]. The length of the NBD1 sequence appears to be critical for solubility of the overexpressed protein, since a shorter domain lacking the 11-residue N-terminal hydrophilic sequence was much less soluble than the 254-residue NBD1 (data not shown). This may explain, at least partly, why Kern et al. [42] recovered a quite short 200-residue protein only in inclusion bodies, whereas a longer unfused NBD1, of 346 residues, was soluble after liberation by thrombin cleavage [41]. In contrast, a similar NBD2 length (234–243 residues) was used by all groups. The highly critical conditions of solubility might therefore be responsible for the failure to ob-

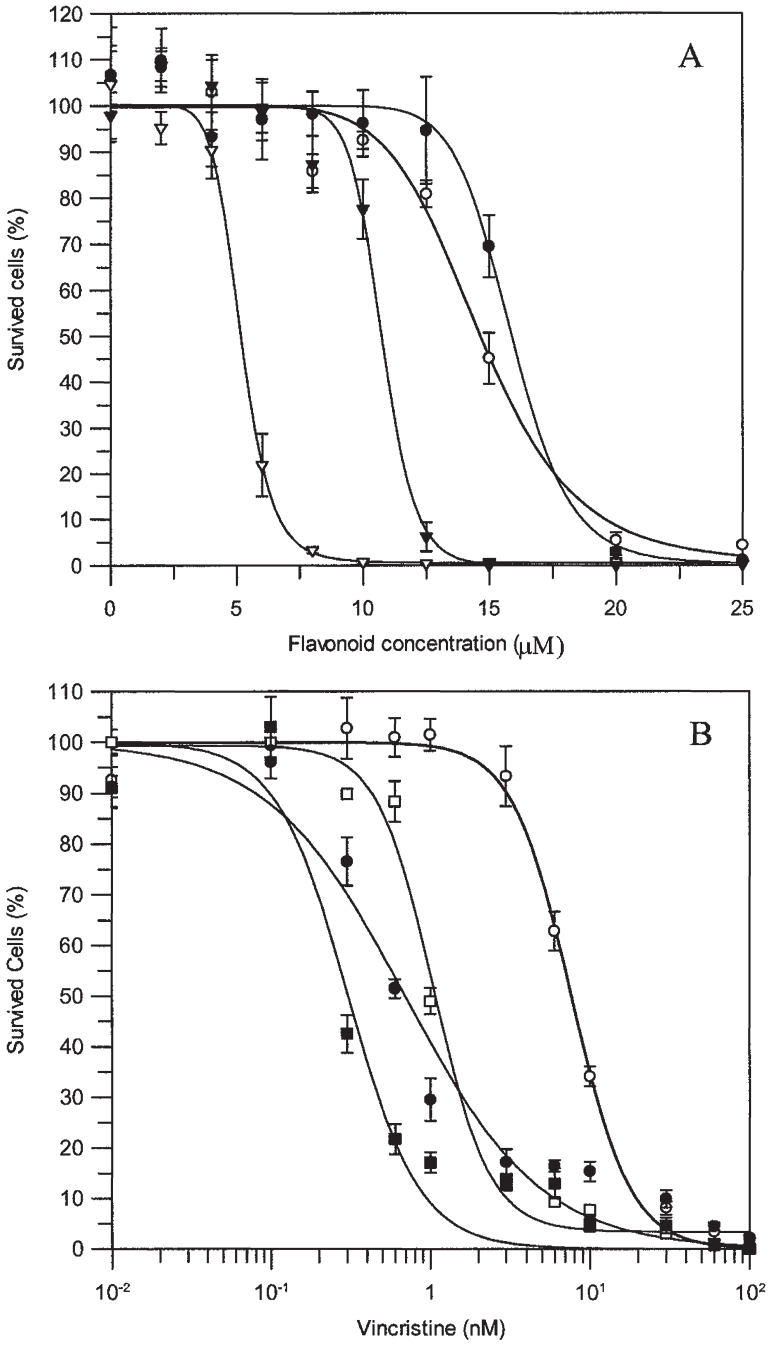

Figure 7. Lower intrinsic cytotoxicity of DHS than 8-geranyl-DHS, and ability to chemosensitize cell growth. (*A*) Intrinsic cytotoxicity: the different cell lines were grown for 4 days in the presence of increasing concentrations of either DHS [control cells (open circle) ; MRP1-transfected cells (closed circle)] or 8-geranyl-DHS [control cells (open inverted triangle); MRP1-transfected cells (closed inverted triangle)]. Remaining living cells were estimated by an MTT test (see Materials and methods). Points are the mean of quintuplicate wells; error bars are the standard deviation. (*B*) DHS-induced reversion of cellular resistance to vincristine: MRP1-transfected BHK-21 cells were grown in the presence of the indicated vincristine concentrations in the absence (open circle), or the presence of a sub-cytotoxic 12.5 μM concentration of DHS (closed circle). The same experiments were performed on control BHK-21 cells, in the absence (open square), or the presence of 12.5 μM DHS (closed square). Points are the means of quintuplicate wells; error bars are the standard deviation.

Table 3. Modulation of the resistance of MRP1-transfected BHK-21 cells to vincristine by DHS, isoprenylated derivatives, and MK571.

Modulator		IC_{50} of control cells (nM)	IC_{50} of transfected cells (nM)	Resistance factor	Reversion (%)
DHS	0 µM	1.05 ± 0.09	7.69 ± 0.39	7.32	
	5 µM	0.505 ± 0.024	2.79 ± 0.34	5.52	24.6
	10 µM	0.34 ± 0.019	1.11 ± 0.16	3.26	55.6
	12.5 µM	0.305 ± 0.06	0.69 ± 0.19	2.26	69.2
6-Prenyl-DHS	0 µM	1.026 ± 0.07	7.46 ± 0.36	7.27	
	10 µM	0.237 ± 0.02	1.81 ± 0.24	7.63	none
6-Geranyl-DHS	0 µM	3.6 ± 0.1	18.0 ± 0.48	4.99	
	7 µM	0.91 ± 0.02	5.65 ± 0.25	6.24	none
8-Geranyl-DHS	0 µM	1.86 ± 0.21	9.35 ± 0.7	5	
	3.5 µM	0.44 ± 0.02	2.6 ± 0.43	5.9	none
8-Prenyl-DHS	0 µM	4.94 ± 0.22	25.6 ± 1.16	5.18	
	2 µM	2.48 ± 0.07	22.1 ± 0.73	8.9	none
MK571	0 µM	1.80 ± 0.19	11.0 ± 0.79	6.12	
	0.5 µM	1.60 ± 0.13	7.88 ± 1.87	4.91	19.8
	1.5 µM	1.17 ± 0.084	4.37 ± 0.45	3.75	38.7

Cell growth experiments in the presence of vincristine, and with or without modulator at indicated concentrations, were performed as described in Materials and methods. The resistance factor was calculated by dividing the IC_{50} of the MRP1-transfected BHK-21 cells by the IC_{50} of the control cells, and allowed the determination of the extent of reversion.

tain soluble NBD2 either by direct overexpression [42] or after thrombin cleavage [41], whereas it was successfully obtained in a cytosolic fraction from insect cells [9, 43]. NBD2 indeed appears here to be intrinsically much less soluble than NBD1. The alkaline pH (8.5) used during lysis and purification steps appears to be critical, since a threefold less soluble NBD2 protein was obtained at neutral pH (7.5) in our system. The two hexahistidine-tagged NBDs prepared here exhibited very low, if any, ATPase activity. A slightly higher activity was reported for fusions with either maltose-binding protein or gluthathione S-transferase, but contradictory results were obtained with respect to vanadate and N-ethylmaleimide inhibitions [41, 42, 44]. In addition, no differential behavior of NBDs could be found [41, 42], whereas the two ATP sites have clearly distinct roles within the whole MRP1 transporter; this was certainly due to the lack of cooperativity [12] which requires interactions between the two NBDs. Consequently, the ATPase activity of isolated NBDs is not suited to monitor events occurring within the entire MRP1. Our studies have therefore focused on nucleotide binding in order to characterize the interaction of flavonoids within NBDs. The high-affinity binding observed, in the micromolar concentration range, for the hydrophobic TNP derivative of ATP was comparable to that found previously with NBDs fused to maltose-binding protein [42], as well as other NBDs from P-glycoproteins [25, 36, 37] or CFTR [45, 46]. The millimolar dissociation constants for ATP, which is able to almost completely displace bound TNP-ATP, are also in the same range as K_m values for ATP hydrolysis by either recombinant NBDs [41, 42, 44] or full-length MRP1 [39, 47]; this further confirms that TNP-ATP indeed binds to the ATP site.

Direct interaction of flavonoids with NBDs and interference with the ATP site: preferential binding to NBD1 upon isoprenylation

The quenching of recombinant protein intrinsic fluorescence constitutes a convenient approach to monitor direct interactions of flavonoids, as shown previously with NBDs from other ABC transporters [24, 25, 48]. MRP1 NBDs are shown here for the first time to bind a series of flavonoids, with the following binding affinity order: flavonols (quercetin, galangin) > flavones (apigenin), flavanones (naringenin) > isoflavones (genistein). Oxidative coupling of positions 3′ and 4′ with a monolignol unit in DHS produced a marked increase in binding affinity with respect to galangin and quercetin, which was much higher than in the case of P-glycoprotein NBD2 [48]. The efficient prevention by ATP of DHS binding to NBD1, with a millimolar dissociation constant similar to that obtained from TNP-ATP displacement experiments, suggests that DHS binds to, or in very close proximity to the ATP site within NBD1, although a distant binding-induced conformational change could not be completely excluded. A mutually exclusive binding between flavonols and ATP derivatives was also observed with P-glycoprotein NBD2 in photoaffinity labeling experiments with TNP-8N_3-ATP [49]. Hydrophobic derivatives of DHS, with a prenyl or geranyl substituent at position 6 or 8, bound with higher affinity than DHS to MRP1 NBDs, suggesting the presence of a hydrophobic inter-

acting region, located outside the ATP site, since ATP was not able to prevent binding of these isoprenylated derivatives. A similarly hydrophobic region, assumed to bind modulatory hydrophobic analogues of steroids, was found in NBDs of P-glycoproteins [24, 25, 31] and in yeast Pdr5p [50]. In contrast, the binding of DHS to NBD2 only partly overlapped the ATP site, since a much lower antagonism was produced by ATP, as compared to NBD1. In addition, the increase in affinity for MRP1 NBDs due to isoprenylation was much higher at NBD1 than at NBD2, indicating either a less hydrophobic region or a steric/conformational hindrance in NBD2. The 13-residue sequence ^{1357}NIAKIGLHDLRFK^{1369}, which constitutes a major alignment difference between the two recombinant NBDs, might be involved, at least partly, in this differential behavior, by preventing access to a hydrophobic-interacting region. The overall results indicate the absence of apparent differences between the two ATP sites of isolated NBDs, consistent with the similar ATPase activity of other recombinant NBDs [41, 42]. However, differential properties appear to concern the immediate proximity of these sites, probably involving a hydrophobic region rendered more accessible within NBD1 due to the absence of the 13-residue sequence. These differences might be related to the distinct roles, either catalytic or regulatory, of the NBDs in the branch C of ABC transporters.

Increase of vanadate-induced 8-N_3-ADP trapping and inhibition of LTC_4 transport

A significant increase in vanadate-induced 8-N_3-ADP trapping by MRP1 is produced upon drug binding [10, 20], which is correlated with stimulation of ATPase activity [51]. A similar correlation has been more widely documented for P-glycoprotein with a series of substrates/modulators, whether they are transported or not [20, 52]. The 122% increase in vanadate-induced 8-N_3-ADP trapping by MRP1 observed here with DHS is much higher than that produced by either doxorubicin [10] or LTC_4 [20], and slightly higher compared to quercetin [20]. It is therefore likely due to interaction of the flavonoid with modulator/drug-binding sites. The higher increase produced by DHS compared to prenylated derivatives might be due to the ability of DHS to mimic ATP through interaction with the NBD1 ATP site, promoting hydrolysis and vanadate-induced 8-N_3-ADP trapping at NBD2 [12]. The inhibition of LTC_4 transport is not related to binding to the ATP site, since it was also observed with prenylated derivatives that do not bind to the ATP site. In addition, the presence of 4 mM MgATP, used to drive energy for LTC_4 transport by the whole transporter, displaced DHS from the NBD1 ATP site, as shown in figure 5A with the recombinant domain. The inhibition of transport is therefore due to DHS binding to the membrane modulator/drug-binding sites, which is consistent with the similar effects produced here by MK571, a structural homologue of drug substrate LTC_4. Interestingly, quercetin was also shown to bind to the drug-binding site of P-glycoprotein, competitively with Hoechst 33342 [53]. The present 4- to 12-fold decrease in inhibition efficiency of LTC_4 transport by MRP1 and its dependency on GSH upon isoprenylation of DHS might be related to increased hydrophobicity, since qualitatively similar, although quantitatively weaker, effects were observed when comparing the relatively more hydrophobic apigenin to kaempferol and quercetin [20].

Flavonoid isoprenylation favors transport at the expense of MDR reversion

The higher intrinsic cytotoxicity of isoprenylated derivatives to control cells compared to MRP1-transfected ones indicates that the latter exhibit some cross-resistance, with resistance index values up to 2.6, suggesting that the more hydrophobic isoprenylated derivatives might be transported by MRP1. This agrees with the GSH-dependent inhibition of LTC_4 transport by isoprenyl derivatives, and suggests GSH cotransport, as this is the case with hydrophobic drugs such as vincristine and daunorubicin [3, 4]. Interestingly, apigenin, the most hydrophobic among flavonoids tested, was also found to exhibit a GSH-dependent inhibition of LTC_4 transport, and shown to be the most efficient in stimulating radioactive GSH transport [20], and to inhibit the GSH-induced stimulation of photolabeling by a tricyclic isoxazole derivative [14]. The fact that isoprenylated derivatives of DHS are not able to induce any reversal of MDR is also consistent with the lack of effect reported for apigenin [20]. In contrast, the absence of cross-resistance for DHS, as reflected by the comparable cytotoxity of control and MRP1-transfected cells, suggests that DHS was not or much less transported, consistent with the lack of GSH dependency of its strong inhibition on LTC_4 transport. Similarly, using a series of steroid derivatives with P-glycoprotein, the best transported compounds were shown to be poor inhibitors whereas the non-transported progesterone highly inhibited vinblastine transport [54]. The strong reversal of MDR, monitored here by chemosensitization of MRP1-transfected BHK-21 cell growth to vincristine, indicates that DHS indeed behaves as a modulator. It appeared even more potent than MK571 since, despite the fact that the latter was effective at 1.5 µM in producing 38.7% reversion, its sixfold higher intrinsic cytotoxicity than DHS in our cellular system limited its use at high concentrations. In contrast, DHS could be used at higher concentrations, enabling greater reversion. The intrinsic cytotoxicity of MK571 was at least tenfold higher in our control BHK-21 cells than in lung cancer GLC4 cells [5] and leukemia CEM cells [55]. A high dependency on cell line was also reported for another inhibitor, GF109203X, which was able to efficiently re-

verse resistance to vincristine in both drug-selected HL60/AR cells and GLC4/ADR cells [56] but did not produce any effect in transfected (T5) HeLa cells [57]. The fact that only very few compounds, such as DHS in transfected BHK-21 cells, and quercetin in transfected (T5) HeLa cells [20], are able to produce cell growth chemosensitization, among a series of flavonoids, in contrast with the stronger effects on substrate transport, suggests that reversion involves a complex mechanism, possibly related to the coexistence and proximity of modulator- and substrate-binding sites.

Acknowledgements. We thank J.-C. Cortay (Lyon, France) for the pT7-7/6His plasmid, and M. Kool and P. Borst (Amsterdam, The Netherlands) for the human 5508-bp MRP1 cDNA. This work was supported by the CNRS, the University Claude-Bernard of Lyon (UMR5086), the Région Rhône-Alpes (EURODOC Program) (D. T.), the Ligue Nationale contre le Cancer (comités de la Loire (D. T.) et du Rhône), the Association pour la Recherche sur le Cancer (No. 4631), the French Ministry of Education (M. M.), and the National Cancer Institute, National Institute of Health (No. CA89078 and DK51619).

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