Correlation between the Affinity of Flavonoids Binding to the Cytosolic Site of *Leishmania tropica* Multidrug Transporter and Their Efficiency To Revert Parasite Resistance to Daunomycin[†]

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Received October 14, 1998; Revised Manuscript Received December 3, 1998

ABSTRACT: The C-terminal nucleotide-binding domain (NBD2) of a P-glycoprotein-like transporter, encoded by the *ltrmdr1* gene in *Leishmania tropica* and involved in parasite multidrug resistance (MDR), was overexpressed in *Escherichia coli* as a hexahistidine tagged protein and purified. The *L. tropica* recombinant domain efficiently bound fluorescent derivatives of ATP, the hydrophobic steroid analogue RU 486, and different classes of flavonoids with the following efficiency: flavone > flavanone > isoflavone > glucorhamnosyl-flavone > chromone. The affinity for flavones was dependent on the presence of hydroxyl groups at positions 5 and 3 and was further increased by a hydrophobic 1,1-dimethylallyl substituent at position 8. When flow cytometry was used to measure daunomycin accumulation in a MDR *L. tropica* line, a reversing effect was observed with flavones such as dimethylallyl-kaempferide at low concentration or apigenin at higher concentration, but neither with the glucorhamnosyl derivative rutin nor with the isoflavone genistein. The in vivo reversing effect of dimethylallyl-kaempferide was correlated to a high inhibition of MDR cell growth in the presence of daunomycin. The results suggest that flavone inhibition of both daunomycin efflux and parasite growth in the presence of the drug correlates to direct binding of the compound to cytosolic domain of the P-glycoprotein-like transporter.

Protozoan parasites are responsible for some of the most important and prevalent diseases of human and domestic animals, threatening the lives of nearly one-quarter of the human population worldwide. This problem has been considerably complicated by the emergence of resistance to drugs (*1*−*4*). Multidrug resistance (MDR)[1] due to P-glycoprotein (Pgp) is a serious impediment to successful chemotherapy in cancer cells. Pgp is a transmembrane ATP-dependent efflux pump that significantly reduces the intracellular accumulation of anticancer drugs (*5*). Recent evidence has shown that Pgp-like are involved in drug resistance in the protozoan parasites *Plasmodium* (*6, 7*), *Entamoeba* (*8*), and *Leishmania* (*9*−*11*).

Structural analysis of a Pgp-like sequence indicates two homologous halves, each composed of a transmembrane domain, involved in drug efflux, and a cytosolic nucleotide-binding domain (NBD), with the characteristic A and B motifs defined by Walker et al. (*12*), involved in ATP binding and hydrolysis. Both halves are linked by a central, potentially phosphorylatable, "linker region". Numerous modulators or chemosensitizers are known to alter the ability of Pgp to maintain subtoxic intracellular drug concentrations; some examples include calcium-channel blockers such as verapamil, detergents such as Triton X-100, and immunosuppressants such as cyclosporin A. These compounds are known to reverse MDR in cancer cells by competing with drug binding to Pgp (*13*). However, they are themselves effluxed and require high concentrations for an efficient and durable inhibition, which produces undesirable side effects. It therefore remains a need to develop new classes of modulators of Pgp with less toxicity for the host. Since ATPase activity is critical for the transporter functioning, the specific inhibition of ATP binding and hydrolysis within the NBDs should constitute a good tool to revert cell MDR phenotype.

Flavonoids constitute a group of interesting polyphenolic compounds with a wide distribution in fruits and vegetables (*14*), with approximately 1 g of mixed flavonoids present in

[†] This work was supported by the European Community Project INCO-DC (ref IC18-CT96-0028), the Spanish Grants ref PM97/0139 and UE97-0019 (F.G.), and the Convenio CSIC-CNRS between F.G. and A.D.P. (1997−99).

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[1] Abbreviations: ABC, ATP-binding cassette; DMA, dimethylallyl; Gdn-HCl, guanidine hydrochloride; 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; NBD2, C-terminal nucleotide-binding domain; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Pgp, P-glycoprotein; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP.

10.1021/bi982455v CCC: $18.00
Published on Web 01/23/1999

the daily Western diet (*15*). Despite their established chemopreventive activity against tumor cell proliferation and pathogenic parasite growth (*16, 17*), contradictory effects have been reported about a possible role in MDR cancer cells. Indeed, flavonoids were found to either increase adriamycin efflux in HCT-15 colon cells (*18*) or, on the contrary, to inhibit rhodamine 123 efflux in MCF-7 breast cells (*19*). Quercetin was shown to inhibit the ATPase activity of Pgp (*20*) and to either inhibit, in the case of Hoechst 33342, or stimulate, in the case of rhodamine 123, the mediated drug efflux (*21*). The binding of different flavonoids to mouse recombinant Pgp has been recently characterized (*22*). However, no systematic study has ever been performed to compare the binding affinity of a series of flavonoids toward Pgp and the differential effect produced on cellular drug efflux and related MDR phenotype.

The aim of the present work was to overexpress and purify the C-terminal nucleotide-binding domain (NBD2) of a parasite Pgp-like multidrug transporter, encoded by the *ltrmdr1* gene of *L. tropica*, to investigate possible direct interaction with different classes of flavonoids and reversal of the MDR phenotype. The results show that only flavonoids which bind with high affinity to the cytosolic domain of the Pgp-like multidrug transporter are able to both increase daunomycin accumulation in a *L. tropica* line overexpressing the transporter and inhibit the parasite growth in the presence of the drug.

EXPERIMENTAL PROCEDURES

Materials. The pQE−30 plasmid, M15 [pREP4] cells and the Ni^{2+}-nitrilotriacetic acid agarose gel were from Qiagen. Guanidine hydrochloride (Gdn-HCl) was from Sigma. 2′-(3′)-*N*-Methylanthraniloyl-ATP (MANT-ATP) and 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) were obtained as described previously (*23*). IPTG (isopropyl 1-thio-β-D-galactopyranoside) was purchased from Boehringer Mannheim, and HECAMEG [6-*0*-[(*N*-heptylcarbamoyl)methyl]-α-D-glucopyranoside] was from Calbiochem. Daunomycin was purchased from Pharmacia Farmitalia (Barcelona, Spain). RU 486 was obtained and used as previously (*24*), whereas progesterone and megestrol acetate were from Sigma. Commercial flavonoids were from either Aldrich (galangin, 7-hydroxyflavone), Sigma (chrysin, naringenin, quercetin, rutin) or Extrasynthèse, Genay, France (acacetin, apigenin, 3-hydroxyflavone, genistein, kaempferide). The 8-(1,1-dimethylallyl) derivatives of chrysin (*25*), apigenin (*26*), and kaempferide (*27*) were synthesized as described.

Parasite Culture and in Vivo Experiments. The wild-type *L. tropica* LRC-strain was obtained from Dr. L. F. Schnur (Kuvin Center for the Study of Infectious and Tropical Diseases, Jerusalem, Israel). The wild-type line used was a clone, obtained by agar plating as described (*28*). A *L. tropica* line resistant to daunomycin (DNM-R150) was maintained in the continuous presence of 150 μM daunomycin and was used as previously described (*11*). Cells were grown at 28 °C in RPMI 1640-modified medium (Gibco), as detailed (*29*) and supplemented with 20% heat-inactivated fetal bovine serum (Gibco). The growth sensitivities of wild-type and drug-resistant parasites to flavonoids were ascertained as described (*11, 30*).

Construction of the Expression Vectors. The DNA encoding the *L. tropica* multidrug transporter LTRMDR1 was obtained as described (*11*). Synthesis of the DNA encoding C-terminal NBD2 was performed by polymerase chain reaction (PCR). The two primers specifics to *ltrmdr1* and corresponding to NBD2 lying from Asp-1076 to Gln-1304 were 5'-ATGGATCCGACCGCGTGCCTGACG-3' and 5'-ATAAGCTTCTGGTCGCAGTGGCGG-3', respectively. The primers allowed the introduction of *Bam*HI and *Hind*III restriction sites. The conditions for PCR were (i) a first denaturation at 95 °C for 5 min, (ii) 4 cycles each consisting in denaturation at 95 °C for 60 s, annealing at 45 °C for 2 min, and elongation at 72 °C for 60 s, (iii) 30 cycles each consisting in denaturation at 95 °C for 60 s, annealing at 64 °C, and elongation at 72 °C for 1 min, and (iv) a final elongation at 72 °C for 10 min. The PCR product with a size of 687 base pairs was cloned into pGEMT (Promega). The recombinant plasmid was digested by *Bam*HI and *Hind*III, and the insert was ligated into the corresponding sites of a linearized pQE−30 plasmid (Qiagen). *Escherichia coli* DH5α (supE44 lacU169 [ø80lacZ M15] hsdR17recA1 endA1 gyrA 96 thi-1relA1) cells were transformed with the ligation product and grown on agar plates supplemented with ampicillin (50 μg/mL). The pQE30-NBD2 was restriction-mapped and dideoxy sequenced to confirm the expected sequence.

Overexpression, Domain Renaturation, and Protein Purification. *Escherichia coli* M15 [pREP4] cells were transformed with pQE30-NBD2 and grown on agar plates supplemented with ampicillin (50 μg/mL) and kanamycin (25 μg/mL). Transformed cells harboring the appropiate recombinant plasmid were grown at 37 °C in LB medium [1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] at pH 7.5 containing 50 μg of ampicillin/mL and 25 μg of kanamycin/mL, until the absorbance at 600 nm reached 0.5 unit. Expression of the recombinant domain was induced with 0.3 mM IPTG for 4 h at 37 °C. Cells were harvested by centrifugation at 4000*g* for 10 min at 4 °C, and resuspended in 50 mM Tris-HCl, pH 7.0, containing 1 mM ethylenediaminetetraacetic acid, 100 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. The cells were lysed by lysozyme (1 mg/mL) at room temperature for 30 min, and the solution was twice sonicated for 30 s, with 30 s interval between each sonication, by using the small probe on the 40% dute pulse setting. The recombinant protein was unsoluble and recovered as inclusion bodies. The pellet from centrifugation at 30000*g* for 20 min at 4 °C was resuspended in 50 mM Tris-HCl, pH 7.5, containing 1 mM ethylenediaminetetraacetic acid, 0.4 M NaCl, 6 mM $MgCl_2$, and 1% (v/v) Triton X-100. The recombinant domain was then solubilized as recently described (*24*) by using 1.5 M Gdn-HCl with vigorous stirring and incubation at 30 °C for 30 min. The supernatant obtained from centrifugation at 30000*g* for 20 min was quickly diluted with 20 volumes of 50 mM potassium phosphate, pH 8.5, containing 150 mM sodium sulfate, 20% (w/v) glycerol, and 0.01% HECAMEG. After centrifugation at 30000*g* for 20 min, the renatured domain was purified through a Ni^{2+}-nitrilotriacetic acid column equilibrated in 50 mM potassium phosphate, 150 mM sodium sulfate, 10% (w/v) glycerol, 1% (v/v) Triton X-100, and 40 mM imidazole, at pH 8.5. The column was first extensively washed with the same buffer containing 0.7 M NaCl and 2 mM β-mercaptoethanol, and then in the absence of Triton X-100 and NaCl but in the presence of 0.05% (w/v)

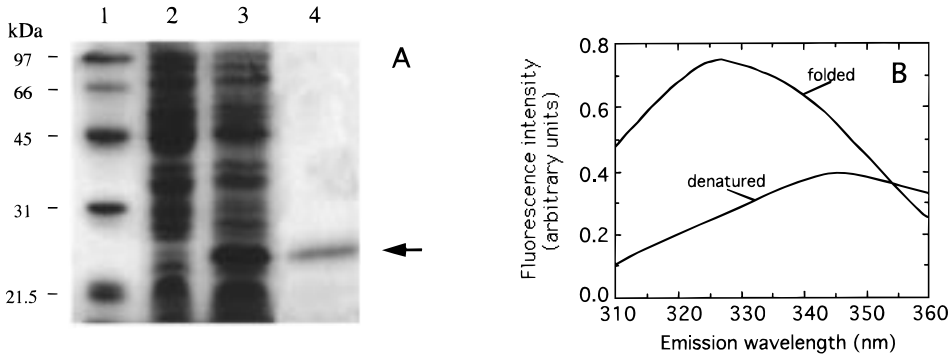

FIGURE 1: Overexpression, purification, and folding of recombinant NBD2. (A) Coomassie-stained SDS−PAGE of total bacteria proteins before (lane 2) or after (lane 3) IPTG induction, and of purified NBD2 first retained on the nickel-chelate resin and then eluted with 200 mM imidazole (lane 4); its position is shown by an arrow on the right. Lane 1 corresponds to molecular weight markers (Bio-Rad) with indicated values. (B) Fluorescence emission spectra of purified NBD2 (1.0 μM) either folded or denatured by 6 M Gnd-HCl, upon excitation at 295 nm.

HECAMEG and 1 mM β-mercaptoethanol. The retained proteins were then eluted with 200 mM imidazole in the same buffer (elution buffer) and analyzed by 12% SDS-polyacrylamide gel electrophoresis as described by Laemmli (*31*). The fractions were pooled and stored in liquid nitrogen. Protein concentration was routinely determined by the method of Bradford (*32*) with the Coomassie blue Protein Assay Reagent kit from Bio-Rad.

Fluorescence Emission Measurements. Experiments were performed at 25.0 ± 0.1 °C using a SLM-Aminco 8000C spectrofluorometer with spectral bandwidths of 2 and 4 nm for excitation and emission, respectively. 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 buffer Raman effect and for dilution.

Fluorescence measurements were performed after dilution of NBD2 (0.2−0.5 μM final concentration) and equilibration for 60 min at 25.0 ± 0.1 °C in 1 mL (final volume) of diluting buffer (50 mM potassium phosphate, pH 8.5, 1 M NaCl, 20% (w/v) glycerol, 0.05% (w/v) HECAMEG, 1 mM β-mercaptoethanol, and adjusting the final imidazole concentration to 10 mM), in the presence of increasing concentrations of nucleotide analogues or dimethyl sulfoxide solutions of either flavonoids or steroids. The NBD2 intrinsic fluorescence was studied by scanning emission in the range of 300−350 nm upon excitation at 288 nm, to minimize the interference of imidazole. The binding of the different compounds was monitored by the quenching of emission fluorescence produced by addition of increasing ligand concentrations. Corrections for inner-filter effect and dimethyl sulfoxide dilution (up to 2% final concentration) were determined under the same conditions by using a mix of *N*-acetyltryptophanamide and *N*-acetyltyrosinamide in the same ratio, 3:7, as tryptophan and tyrosine residues present in NBD2. Curve fitting was accomplished with the Grafit program (Erithacus Software) as detailed previously (*33*).

Drug Accumulation by Flow Cytometry. The ability of wild-type and DNM-R150 lines to accumulate daunomycin from the culture medium was determined as recently described (*11*). The parasites were harvested by centrifugation and washed three times in phosphate-buffered saline (PBS, 1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 130 mM NaCl, 2.6 mM KCl adjusted to pH 7.4). The concentration was adjusted to 10^6 parasites, in 1 mL of PBS containing 0.1% bovine serum albumin, which were incubated with 8 μM daunomycin for 1 h at 28 °C in the presence or absence of flavonoids. Drug efflux was stopped by centrifugation at 4 °C. Parasites were then washed three times in ice-cold PBS before analysis. The intracellular level of daunomycin was estimated by flow cytometry, as described (*11*), in a Beckton Dickinson FacScan apparatus using the fluorescence emission of the drug scanned over the range 540−590 nm. Analysis was gated to include only live, single cells and was based on data acquisition from 10 000 cells.

RESULTS

Overexpression and Purification of the NBD2 Domain from Leishmania tropica Pgp-like Transporter. On the basis of DNA sequence and predictions from hydrophobicity profiles of the LTRMDR1 protein from *L. tropica* (*11*), the two nucleotide-binding domains (NBD1 and NBD2) are assumed to be cytosolic; they both contain the Walker motifs A and B involved in ATP binding and the ABC (ATP-binding cassette) transporter signature. The first aim of the present study was to obtain the cytosolic NBD2 domain as a N-terminal hexahistidine-tagged recombinant protein. The NBD2 protein, as defined from aspartate-1076 to glutamine-1304, contains three tryptophan residues located at positions 1171, 1216, and 1221, between the Walker motifs A and B. For this purpose, the corresponding DNA was obtained by PCR and inserted into the pQE30 vector for overexpression in M15 *E. coli* cells and further purification by affinity chromatography.

Figure 1A shows that the recombinant NBD2, with an apparent molecular mass of 26 kDa consistent with the theoretical value, was highly overexpressed upon IPTG induction (Figure 1A, lane 3) and constituted the main component among total bacterial proteins. The recombinant domain was exclusively recovered as inclusion bodies in the pellet of centrifugation, whatever the overexpression conditions assayed. It was successfully renatured as a soluble protein by a method recently used for the NBD1 domain from mouse Pgp (*24*): solubilization from inclusion bodies by a low Gdn-HCl concentration (1.5 M) and renaturation by quick dilution with 20 volumes of phosphate buffer at pH 8.5, containing glycerol and high salt concentration. The recombinant domain was then loaded onto a nickel-agarose column for final purification, the retained fraction was eluted

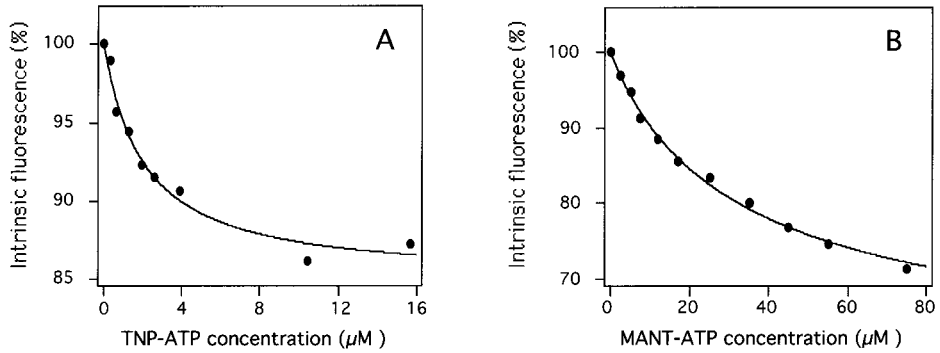

FIGURE 2: Interaction of recombinant NBD2 with ATP analogues as monitored by quenching of the protein intrinsic fluorescence. The binding of TNP-ATP (A) or MANT-ATP (B) to NBD2 was determined by quenching of the domain intrinsic fluorescence as described under Experimental Procedures. The fluorescence spectrum of 0.5 μM NBD2 was recorded upon excitation at 288 nm and corrected for buffer contribution and nucleotide analogue inner-filter effect. The concentration-dependent binding was analyzed by the increasing quenching of NBD2 intrinsic fluorescence, as determined by spectrum integration from 300 to 350 nm.

Table 1: Effects of Flavone Substitution on the Affinity Binding to NBD2[a]

	ring C	ring A			ring B		
flavones	[3]	[5	7	8]	[4′]	K_d (μM)	maximal quenching (%)
7-OH-flavone			OH			83.7 ± 15.5	73.7 ± 7.7
3-OH-flavone	OH					18.7 ± 3.2	99.7 ± 8.7
chrysin		OH	OH			17.6 ± 5.9	81.3 ± 8.6
galangin	OH	OH	OH			9.2 ± 1.0	75.9 ± 2.1
apigenin		OH	OH		OH	15.7 ± 1.0	91.8 ± 1.7
acacetin		OH	OH		OCH_3	20.9 ± 2.0	88.6 ± 2.7
kaempferide	OH	OH	OH		OCH_3	13.6 ± 2.3	79.2 ± 3.9
DMA-chrysin		OH	OH	1,1 DMA		1.4 ± 0.2	75.4 ± 2.7
DMA-apigenin		OH	OH	1,1 DMA	OH	0.7 ± 0.1	70.1 ± 1.6
DMA-kaempferide	OH	OH	OH	1,1 DMA	OCH_3	0.7 ± 0.1	80.5 ± 2.1

[a] The NBD2 domain was incubated, under conditions of Figure 3B, with differently subtituted flavones.

by competition with 200 mM imidazole (Figure 1A, lane 4).

The intrinsic fluorescence spectrum of purified renatured NBD2, due to its three tryptophan residues, exhibited a maximum of fluorescence emission at 327 nm which reveals a hydrophobic environment characteristic of a folded protein (Figure 1B). A considerable red shift in the peak (about 20 nm) was produced when the domain was denaturated for at least 3 h with 6 M Gdn-HCl. Addition to folded NBD2 of a fluorescent ATP analogue, such as either TNP-ATP or MANT-ATP (Figure 2), produced a significant quenching of the domain intrinsic fluorescence upon excitation at 288 nm. The quenching was dependent on the nucleotide analogue concentration, giving an apparent dissociation constant (K_d) of 1.9 ± 0.4 μM for TNP-ATP (Figure 2A) or 35.9 ± 7.9 μM for MANT-ATP (Figure 2B).

Interaction of NBD2 with Steroids and Flavonoids. Incubation of purified recombinant NBD2 with the hydrophobic antiprogestin RU 486 produced a high quenching of the domain intrinsic fluorescence (Figure 3A) with a K_d of 7.8 ± 1.4 μM, whereas a lower affinity binding was observed with megestrol acetate and progesterone. These results support the presence of a hydrophobic-binding region in *Leishmania* NBD2 in addition to the ATP-binding site, as recently proposed for mouse Pgp (*24*).

The binding of flavonoids to NBD2 was also monitored by quenching of the protein intrinsic fluorescence. Figure 3B indicates that the interaction was strongly dependent on the class of flavonoid, with the flavone apigenin, whose chemical structure is shown, being the most efficient. A much lower affinity binding was observed for the flavanone naringenin, where the 2,3-double bond of ring C is reduced, and for the isoflavone genistein, where ring B is branched at position 3, instead of 2 in the other cases. Complete replacement of ring B by a methyl group at position 2 and an acetyl group at position 3, in chromone, almost completely abolished the binding. Glucorhamnosylation of quercetin, another flavone with two additional hydroxyl groups at positions 3 and 3′, to give rutin, also markedly altered the binding.

The binding affinity for flavones, which produced a high maximal quenching (>70%), was affected by both hydrophilic and hydrophobic substituents (Table 1). A hydroxyl group at position 3 or 5, close to the ketone group at position 4, produced a 4- to 5-fold higher affinity for binding than that at position 7, which was not significantly different from unsubstituted flavone. The effects of the two important hydroxyl groups appeared additive in galangin (K_d = 9.2 μM). The presence of either hydroxyl or methoxyl group at position 4′ of ring B had no marked effect in apigenin or acacetin, and the positive effect of a hydroxyl group at position 3 was observed again with kaempferide as compared to acacetin. A further 20-fold increase in binding affinity was produced by addition of the hydrophobic 1,1-dimethylallyl (DMA) group at position 8 of ring A, as also observed for chrysin, apigenin, and kaempferide, a K_d in the nanomolar range being obtained for the two last derivatives.

Table 2 shows that the binding of DMA-kaempferide partly overlapped the two binding sites for ATP and RU 486, as the binding was antagonized by either ATP or RU 486

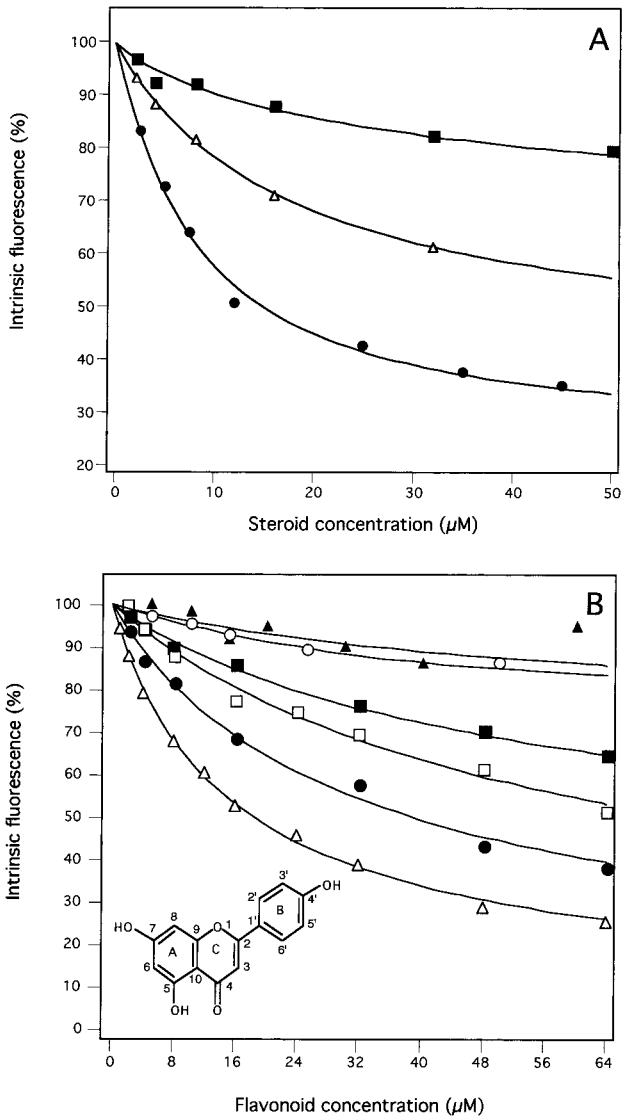

FIGURE 3: Interaction of recombinant NBD2 with steroids and flavonoids. (A) The intrinsic fluorescence of recombinant NBD2 was measured under conditions of Figure 2, in the presence of increasing concentrations of either RU 486 (●), megestrol acetate (△), or progesterone (■) as dimethyl sulfoxide solutions. (B) Different classes of flavonoids were assayed instead of steroids: apigenin (△), a 5,7,4′-trihydroxy flavone whose chemical structure is shown at the bottom of the figure, naringenin (□), a flavanone corresponding to apigenin with reduced 2,3-double bound, genistein (■), an isoflavone corresponding to apigenin with ring B branched on position 3 of ring C, quercetin (●), a 3,5,7,3′,4′-pentahydroxy flavone, rutin (○), a 3-glucorhamnosyl derivative of quercetin, or chromone (▲), lacking complete ring B.

which highly increased the K_d value. The binding of ATP and RU 486 occurred at separate binding regions since ATP did not antagonize RU 486 binding, as opposed to DMA-kaempferide.

Modulation by Flavonoids of Daunomycin Accumulation in a Daunomycin-Resistant Leishmania tropica Line. The resistance to daunomycin in a MDR *L. tropica* line was related to the failure to accumulate the drug, which is mainly due to overexpression of a Pgp-like transporter involved in drug efflux (*11*). To evaluate whether the binding of flavonoids to the NBD2 cytosolic domain of the Pgp-like transporter correlates with increased daunomycin accumulation in resistant parasites, daunomycin uptake was measured by using laser flow cytometry after incubating wild-type and

Table 2: Antagonism Against Ligand Binding to NBD2 by Preincubation with Other Ligands[a]

ligand	preincubation with	K_d for the ligand (μM)
RU 486	none	7.8 ± 1.4
	10 mM ATP	7.6 ± 1.4
	1.5 μM DMA-kaempferide	24.9 ± 3.2
DMA-kaempferide	none	0.7 ± 0.1
	10 mM ATP	1.5 ± 0.2
	20 mM ATP	1.8 ± 0.3
	8.2 μM RU 486	2.4 ± 0.7

[a] The NBD2 domain was preincubated for 60 min in the presence of a fixed concentration of either ATP, DMA-kaempferide, or RU 486 and then assayed for the binding of RU 486 or DMA-kaempferide at increasing concentrations as above.

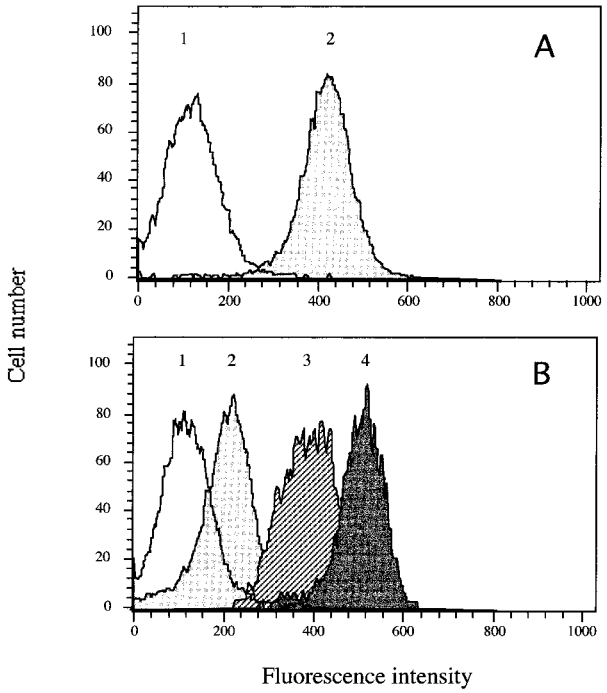

FIGURE 4: Cellular accumulation of daunomycin on *L. tropica* lines. Fluorescence intensity histograms were obtained by flow cytometry after incubation for 1 h at 28 °C of wild-type (A) and daunomycin-resistant (B) parasites without any drug (profiles 1), or with either 8 μM daunomycin alone (profiles 2), 8 μM daunomycin plus 200 μM apigenin (profile 3), or 8 μM daunomycin plus 50 μM DMA-kaempferide (profile 4). A total of 10 000 cells were counted for each histogram. The experiments were repeated three times and gave very similar profiles as the typical experience shown here.

daunomycin-resistant parasites with 8 μM daunomycin with or without flavonoids. Fluorescence intensity histograms of wild-type (A) and resistant parasites (B) incubated without drug are displayed in Figure 4 (profiles 1). The peak of fluorescence distribution after incubation with daunomycin was significantly shifted to the right in the wild-type with respect to the resistant parasites (profiles 2). The resistant line exhibited a significantly lower daunomycin accumulation expressed as mean fluorescent channel (m = 222.5) compared to that of wild-type (m = 421.2). Treatment of the resistant cells with either 50 μM DMA-kaempferide or 200 μM apigenin resulted in a significant shift of the fluorescence peak to the right, showing an increased daunomycin accumulation in the resistant line with m = 470.8 (profile 4) and 373 (profile 3), respectively. In contrast, very limited effects were produced by flavonoids in the wild-type lines

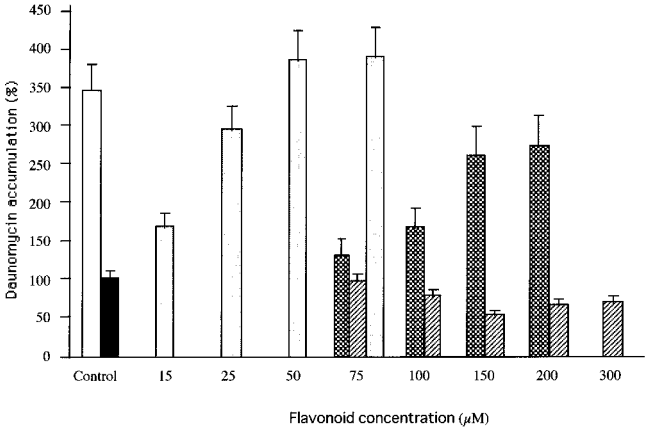

FIGURE 5: Differential modulation by flavonoids of daunomycin accumulation in a resistant *L. tropica* line. Wild-type (open bar) and resistant (solid bar) parasites incubated with 8 μM daunomycin for 1 h at 28 °C served as control of daunomycin accumulation. Resistant parasites were incubated with 8 μM daunomycin in the presence of increasing concentrations of modulators: DMA-kaempferide (shaded bars), apigenin (squared bars), or rutin (hatched bars). Data are expressed as percent daunomycin accumulation in the presence as compared to the absence of modulator. Each point is the mean ± standard deviation of duplicate determinations from three independent experiments.

(not shown). The flavonoids effects on daunomycin accumulation in daunomycin-resistant cells were concentration-dependent, with DMA-kaempferide being more effective than apigenin (Figure 5). DMA-kaempferide at 25 μM was even more efficient on daunomycin accumulation ($m = 393.3$) than either cyclosporin A or verapamil at 75 μM ($m = 328$ or 371, respectively) (not shown here). In contrast, rutin did not increase at all daunomycin accumulation (Figure 5), which correlates with its low affinity binding to recombinant NBD2 (Figure 3B). No effect was either observed for the isoflavone genistein (data not shown). On the contrary, DMA-kaempferide, which binds with high affinity to NBD2, completely reversed at 50 μM, daunomycin accumulation in resistant parasites (Figure 5).

The in vivo reversing effect of DMA-kaempferide was further studied on the growth of resistant parasites in the presence of daunomycin by comparison with wild-type parasites in the absence of drug (Figure 6). The results show that a 48 h incubation of resistant parasites in a medium containing 150 μM daunomycin and 50 μM DMA-kaempferide produced more than 80% inhibition of the growth, as compared to the same conditions in the absence of flavonoid. This contrasts with the very limited effect produced by the flavonoid on the wild-type line, around 5 and 20% inhibition of growth for 25 and 50 μM DMA-kaempferide, respectively, which could correspond to some low binding to other cellular targets. The results strongly suggest that the in vitro binding of flavonoids to recombinant NBD2 correlates with their in vivo ability to inhibit drug efflux by, and growth of, resistant parasites.

DISCUSSION

The main original finding of this paper is that flavone binding to Pgp-like transporter of *L. tropica* is responsible for inhibition of its drug-efflux activity and reversal of multidrug resistance of the parasite cells.

Overexpression and Purification of the NBD2 Domain of Leishmania tropica Multidrug Transporter and Interaction with Flavonoids. Overexpression and purification of the NBD2 cytosolic domain from the *Leishmania* Pgp-like transporter has allowed us to initiate the screening of a series of flavonoids, to establish structure−activity relationships in the context of a rational drug-design approach with the final aim to revert MDR phenotype in protozoan parasites. The amino acid sequence 1076−1304 of the multidrug transporter from *Leishmania* as a putative NBD was chosen after a comparison with other ABC transporters. The recombinant NBD2 domain from *Leishmania* appeared to be more hydrophobic than NBDs from mouse Pgp prepared as fusion proteins with either glutathione *S*-transferase or a N-terminal hexahistidine tag (*23*, *24*, *34*) and was recovered as insoluble inclusion bodies whatever the overexpression conditions used. The protein was successfully renatured by a procedure involving solubilization with 1.5 M Gdn-HCl and quick dilution with 20 volumes of phosphate buffer, at alkaline pH, containing glycerol and high salt concentration, as recently described for an extended NBD1 domain from mouse Pgp (*24*). The presence of the uncharged detergent HECAMEG also appeared critical for solubilization. Evidence that the domain reached a folded and stable conformation was provided by the intrinsic fluorescence spectrum with a low wavelength for maximal fluorescence, 327 nm, which was highly red-shifted by denaturation with 6 M Gdn-HCl. This suggests that tryptophan residues located at positions 1171, 1216, and 1221, between the A and B Walker's motifs, are in a nonpolar environment as also observed for most proteins in their native state (*35*). Conclusive observation that NBD2 recovers a native-like conformation comes from

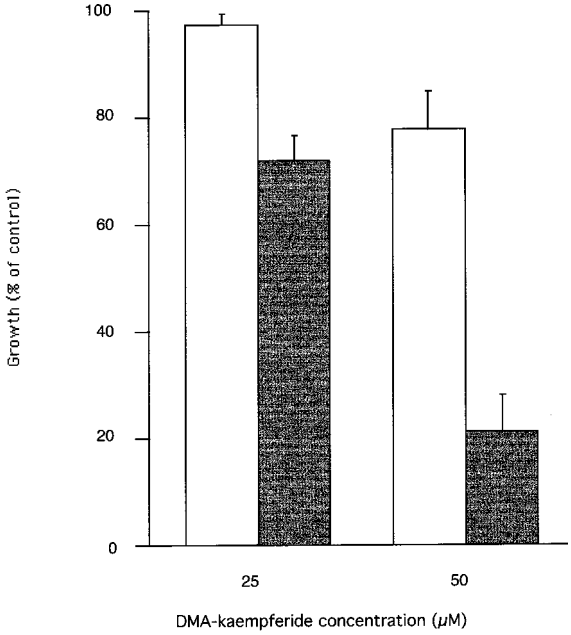

FIGURE 6: In vivo reversing effect of DMA-kaempferide in a daunomycin-resistant *L. tropica* line. Cell growth of either wild-type or resistant parasites was determined after 48 h incubation at 28 °C. Open bars, represent wild-type parasites grown in the presence of different concentrations of DMA-kaempferide. Solid bars, represent resistant parasites grown in the presence of 150 μM daunomycin together with the same concentrations of DMA-kaempferide. The results are expressed as the percentage of growth observed in both cell lines by reference to that observed in the absence of flavonoid (control cells). The means ± standard deviation of duplicate measurements of three independent experiments are shown.

its capacity to bind fluorescent analogues of ATP, namely MANT-ATP and TNP-ATP, with K_d values similar to those previously reported for other purified NBDs from different ABC transporters (*23*, *24*, *33*, *34*, *36*).

Recombinant NBD2 from the protozoan parasite *Leishmania* exhibits a series of structural characteristics: (i) a preference for hydrophobic steroids binding, RU 486 > megestrol acetate > progesterone; (ii) a preference for flavonoids binding, flavone > flavanone > isoflavone > glucorhamnosyl-flavone > chromone; (iii) distinct binding sites for ATP and steroids; and (iv) antagonism by both ATP and steroids against flavonoid binding. All of these results indicate that, similarly to mouse NBDs (*22*), the *L. tropica* multidrug transporter NBD2 contains vicinal ATP and steroid-like binding sites which are partly overlapped by the bound flavonoids. Additional findings concern the high increase in affinity (20-folds) produced by addition of the hydrophobic 1,1-DMA substituent at position 8, as observed in chrysin, apigenin, and kaempferide. This could indicate a strengthening of interaction at the steroid-like hydrophobic binding site.

Ability of High-Affinity Flavones To Inhibit Cellular Drug Efflux and To Revert Multidrug Resistance. The affinity binding order observed for flavonoids toward the recombinant NBD2 appears to directly correlate their ability to increase daunomycin accumulation in a MDR *Leishmania* line: DMA-kaempferide > apigenin > genistein, rutin. Indeed, 50 μM DMA-kaempferide was found to reverse daunomycin accumulation to a higher extent than 200 μM apigenin, whereas genistein and rutin, with low affinity for quenching NBD2, did not exhibit any ability to revert daunomycin accumulation in the resistant parasites. Additional compounds would be required to strengthen the correlation and to further characterize the critical structure−activity relationships. In vivo experiments of MDR reversal by using DMA-kaempferide showed that the substituted flavone also produced a dose-dependent inhibition of the growth of resistant parasites. The present results are consistent with the reversing effects of quercetin and its hydrophobic 3′,4′,7-trimethoxy derivative in a human MDR MCF-7 breast-cancer cell line (*19*). However, it cannot be excluded that the residual growth of the resistant cells insensitive to 50 μM DMA-kaempferide could be related to other, multidrug transporter independent, mechanisms of resistance such as modifications of cytoplasmic pH, altered detoxification pathways or modification of the membrane permeability, as previously described (*11*).

The flavone efficiency to revert multidrug resistance appears directly related to its dual antagonism against ATP binding (*22*, *this work*), which would lead to inhibition of ATPase activity (20), and against efflux of drugs such as daunomycin (*this work*) or Hoechst 33342 (*21*). Despite the above evidences for a direct interaction of flavonoids with NBDs of the transporter, it cannot be completely excluded that other alternatives such as interaction with membrane phospholipids, altering the lipid packing density and the diffusion rate of the drug (*37*), or modulation of the multidrug transporter expression, as previously proposed (*38*), might contribute to the observed reversal of multidrug resistance.

In conclusion, our data indicate that the recombinant NBD2 cytosolic domain from *Leishmania* multidrug transporter represents a biological material well-suited to screening flavonoids for a rational drug-design of inhibitors directed against functioning of Pgp or homologous multidrug transporters in parasites. It is interesting to speculate whether similar flavonoids would be active against the PfMDR1 multidrug transporter in *Plasmodium falciparum*.

ACKNOWLEDGMENT

We thank Pilar Navarro for the help in the parasite culture. We also acknowledge Pharmacia Farmitalia (Barcelona, Spain) for providing the daunomycin used in this study and Dr. A. W. Coleman for improving the English of the manuscript. J.M.P.-V. was recipient of a fellowship from the Junta de Andalucia, Spain, and G.C. and G.D. of fellowships from the Ligue Nationale contre le Cancer, France.

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BI982455V