High-Affinity Binding of Silybin Derivatives to the Nucleotide-Binding Domain of a *Leishmania tropica* P-Glycoprotein-Like Transporter and Chemosensitization of a Multidrug-Resistant Parasite to Daunomycin

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In order to overcome the multidrug resistance mediated by P-glycoprotein-like transporters in *Leishmania* spp., we have studied the effects produced by derivatives of the flavanolignan silybin and related compounds lacking the monolignol unit on (i) the affinity of binding to a recombinant C-terminal nucleotide-binding domain of the *L. tropica* P-glycoprotein-like transporter and (ii) the sensitization to daunomycin on promastigote forms of a multidrug-resistant *L. tropica* line overexpressing the transporter. Oxidation of the flavanonol silybin to the corresponding flavonol dehydrosilybin, the presence of the monolignol unit, and the addition of a hydrophobic substituent such as dimethylallyl, especially at position 8 of ring A, considerably increased the binding affinity. The in vitro binding affinity of these compounds for the recombinant cytosolic domain correlated with their modulation of drug resistance phenotype. In particular, 8-(3,3-dimethylallyl)-dehydrosilybin effectively sensitized multidrug-resistant *Leishmania* spp. to daunomycin. The cytosolic domains are therefore attractive targets for the rational design of inhibitors against P-glycoprotein-like transporters.

Drug resistance has become a major impediment to the treatment of diseases caused by protozoan parasites, which threaten the life of nearly one-quarter of the human population. Among parasitic infections, World Health Organization statistics show that the incidence of leishmaniasis has increased 42-fold between 1985 and 1998 and has become the second leading cause of death (18) worldwide. Chemotherapy remains the only effective way to control infections. The conventional clinical drugs, pentavalent antimonials in the form of Glucantime and Pentostam (reviewed in reference 27), are not very efficient due to their toxicity and the increased appearance of drug resistance (14). ATP-binding cassette (ABC) transporters have been found to be involved in Leishmania species in vitro selected for metal resistance (reviewed in reference 30). The multidrug resistance (MDR) phenotype due to P-glycoprotein (Pgp)-like transporters has been extensively characterized in cancer cells (1, 13) and protozoan parasites (41), including Plasmodium (45) and Leishmania (4, 5, 17) spp. Pgp is an ATP-dependent pump that exports a wide range of drugs from the cell, decreasing their intracellular concentration and preventing their cytotoxic activity. Pgp belongs to the ABC superfamily of transporters. It consists of two homologous halves, each comprising a transmembrane domain involved in drug efflux, and a cytosolic nucleotide-binding domain (NBD) responsible for ATP binding and hydrolysis. Pgp can be inhibited

* Corresponding author. Mailing address: Instituto de Parasitologia y Biomedicina "López-Neyra," Consejo Superior de Investigaciones Cientificas, c/Ventanilla 11, 18001 Granada, Spain. Phone: 34-958-805185. Fax: 34-958-203323. E-mail: gamarro@ipb.csic.es. in vitro by agents such as verapamil and cyclosporine, which compete with drug binding to the transmembrane domains (16). However, most of these inhibitors are also pumped substrates and therefore require high concentrations for effective inhibition. These concentrations produce undesirable side effects. In addition, these classical modulators of drug efflux in cancer cells only poorly sensitize the MDR phenotype in Leishmania parasites (4, 17, 33). Thus, new classes of more specific, nontransported inhibitors of Pgp-like transporters with lower host toxicity need to be developed. Recently, it has been described that NBDs can be the target for inhibitors of Pgp-like transporters (7, 11, 33). Flavonoids, which constitute a wellknown class of natural inhibitors of different ATP-binding proteins (28), with contradictory modulation effects on different MDR cells (8, 15, 33, 37–39), bind to transporter NBDs. They interact with both the ATP-binding site and a vicinal hydrophobic region (7, 9, 33), inhibiting drug efflux and reversing the resistance phenotype of an L. tropica MDR line (33). Their efficient modulation of drug efflux has been correlated with their affinity binding to the transporter cytosolic domain (33).

Silymarin is a mixture of flavanolignans isolated from the medicinal plant *Silybum marianum*, with silybin (or silybinin) (Fig. 1A) as the main component (31). These natural compounds are well-established hepatoprotectants and are used in Europe and Asia for the clinical treatment of liver diseases with different aetiologies (reviewed in references 25 and 32). Silymarin is well tolerated as a therapeutic agent and is largely devoid of adverse effects (25, 32). It has been recently marketed in the United States and in Europe as a nutritional supplement. Silymarin may directly affect cholesterol metabo-



FIG. 1. Chemical structures of silybin and derivatives. (A) The flavanol silybin with a reduced 2,3-bond, a hydroxyl group at position 3 and the monolignol unit (rings D and E) adjacent to ring B. (B) The flavonol DHS with oxidized 2,3-bond and derivatives substituted by 3,3-DMA or geranyl groups at either position 6 (R1) or 8 (R2) of ring A. (C) The flavonol galangin, lacking the monolignol unit, and derivatives substituted at position 8 (R3). (D) The flavone chrysin, lacking both the monolignol unit and the hydroxyl group at position 3, and 8-substituted derivatives (R3).

lism and is therefore considered as a potential hypocholesterolemic (41). In vitro studies indicate that silymarin and silybin may help to prevent and treat breast, prostate, skin and ovarian cancers (32, 36, 46). Silybin also appeared to be synergistic with doxorubicin in a doxorubicin-resistant cell line, probably by inhibiting Pgp function (36). Thus, silybin, either alone or in combination with other cytotoxic drugs, is currently being tested in patients with advanced ovarian cancer (36).

The binding of flavonoids to Pgp (7, 33) and their modulation of drug efflux in an *L. tropica* MDR line are class dependent (33). Flavones and flavonols display better binding affinities than the corresponding isoflavones and flavanones. The present study has tested the ability of the flavanonol silybin, plus its oxidized and hydrophobically substituted derivatives, to bind to purified recombinant C-terminal NBD (NBD2) of a *Leishmania* Pgp-like transporter and to sensitize the MDR phenotype. The oxidation of silybin to its corresponding flavonol and the prenylation of the latter, especially at position 8, dramatically increased the binding affinity for NBD2 and the sensitization of an *L. tropica* MDR line overexpressing the transporter, thereby inhibiting growth in the presence of the daunomycin.

MATERIALS AND METHODS

Chemical compounds. HECAMEG [6-*O*-[(*N*-heptylcarbamoyl)methyl]-α-D-glucopyranoside] was purchased from Calbiochem, daunomycin was obtained from Pharmacia & Upjohn (Barcelona, Spain), and imidazole (catalog reference I 0250) was from Sigma. Commercial flavonoids were obtained from either Aldrich (galangin) or Sigma (chrysin and silybin). 1,1-DMA-chrysin (3), 1,1-DMA-galangin (2) and the derivatives of silybin (26) were synthesized as described. 3,3-DMA-chrysin was from the Natural Products Laboratory collections of compounds.

Synthesis of 8-(3,3-DMA)-galangin. To a stirred solution of 1.2 g of galangin (4.4 mmol) and 1.6 g of tetraethylammonium iodide (6.2 mmol) in 70 ml of 10% aqueous tetramethylammonium hydroxide was added 1 ml of prenyl bromide (8.7 mmol) dropwise at room temperature. After a 90-min reaction, the medium was acidified to pH 1 (with HCl, 1 N) and extracted with ethyl acetate. Isolation of 8-(3,3-DMA)-galangin (0.19 g, 0.6 mmol, 14%) from the ethyl acetate extract was carried out by medium pressure liquid chromatography on a C₁₈ reversed-phase column using a gradient of methanol in water as solvent. The ¹H nuclear magnetic resonance (acetone-d₆, 300 MHz) variables were as follows: δ 12.02 (1H, s, 5-OH), δ 8.30 (2H, m, H-2'+6'), δ 7.55 (3H, m, H-3'+4'+5'), δ 6.40 (1H, s, H-6), δ 5.30 (1H, brt, J = 6.5 Hz, H-2), δ 3.59 (2H, d, J = 6.5 Hz, H-1), δ 1.83 (3H, s, H-5), and δ 1.67 (3H, s, H-4). For electron impact mass spectrometry (EIMS) (70 eV), the *m/z* (rel. int.) values were 338 [M]⁺ (94), 323 (100), 283 (72), and 270 (42). For high-resolution EIMS, the *m/z* value was 338.1155 (as calculated for C₂₀H₁₈O₅ = 338.1154).

Parasite culture and in vitro experiments. The wild-type *L. tropica* LRC strain was a clone obtained by agar plating (19). An *L. tropica* line highly resistant to

daunomycin (DNM-R150) was maintained in the presence of 150 μ M daunomycin and used as previously described (4). This resistant line had an MDR phenotype similar to that of tumor cells, with cross-resistance to several drugs and an overexpressed drug efflux Pgp-like transporter (4). Promastigote forms were grown at 28°C in RPMI 1640-modified medium (Gibco) (20) and supplemented with 20% heat-inactivated fetal bovine serum (Gibco). The growth sensitivity of wild-type and drug-resistant parasites to modulators of drug efflux was ascertained as described earlier (33, 34).

Overexpression, protein purification, and interaction of Leishmania NBD2 with silybin and derivatives. The recombinant NBD2 from Leishmania Pgp-like transporter was overexpressed in E. coli M15 (pREP4) cells and purified by affinity chromatography (33). Fluorescence experiments were performed at 25.0 ± 0.1°C using an SLM-Aminco 8000C spectrofluorimeter with spectral bandwidths of 2 and 4 nm for excitation and emission, respectively. The measurements were corrected for 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. The intrinsic fluorescence of NBD2 (0.2 to 0.5 µM final concentration) was measured in 1 ml of diluting buffer (50 mM potassium phosphate, pH 8.0; 1 M NaCl; 20% [wt/vol] glycerol, 0.05% [wt/vol] HECAMEG; 1 mM β-mercaptoethanol; 10 mM imidazole), with increasing concentrations of silvbin or derivatives dissolved in dimethyl sulfoxide. The emission spectrum was measured in the range of 300 to 350 nm upon excitation at 288 nm (a wavelength which minimized imidazole interference [33, 34]). Ligand binding was monitored by the quenching of emission fluorescence produced upon addition of increasing ligand concentrations. Corrections for the inner-filter effect and dimethyl sulfoxide addition (up to a 2% final concentration) were determined under the same conditions, by using a mixture of Nacetyltryptophanamide and N-acetyltyrosinamide in the same ratio (3:7) as the tryptophan and tyrosine residues present in NBD2. Curve fitting of ligand binding-related fluorescence quenching was analyzed with the Grafit program (Erithacus Software) (12). This allowed the determination of the apparent dissociation constant (K_d) and maximal quenching of fluorescence.

RESULTS

Interaction of NBD2 with silybin and derivatives. Incubation of recombinant NBD2 with the naturally occurring flavanolignan silvbin (Fig. 1A) produced the concentration-dependent quenching of protein intrinsic fluorescence illustrated in Fig. 2A. Detailed analysis of binding (Fig. 2B) gave an apparent dissociation constant (K_d) of 9.2 \pm 1.0 μ M. Oxidation of the 2,3-bond of ring C in silvbin to the corresponding flavonol dehydrosilybin (DHS) (Fig. 1B), gave a fourfold-higher binding affinity for the domain (Fig. 2B, with a K_d of 2.3 \pm 0.2 μM). A further 3.5- to 21-fold increase in binding affinity was produced by addition of hydrophobic substituents (dimethylallyl [DMA] or geranyl) at either position 6 or position 8 of ring A, with K_d values in the nanomolar range (Table 1). The effect was dependent on both the nature and the position of the hydrophobic substituent. Thus, a 3,3-DMA group at position 6 or 8 of ring A increased the binding affinity a further 2.5- to 3-fold more, respectively, than a geranyl group at the same positions (Table 1). In addition, the hydrophobic substitution at position 8 of ring A by either prenyl or geranyl substituent gave twofold-greater affinity than substitution at position 6 (Table 1). Thus, the 8-(3,3-DMA)-DHS derivative gave the highest binding affinity, with a K_d of 0.11 \pm 0.02 μ M. This value was 85-fold less than that obtained with unmodified silybin.

The effect of prenylation at position 8 was further studied within the compounds galangin and chrysin (Fig. 1C and D), lacking the monolignol unit. When compared to DHS, the binding affinity for NBD2 was reduced fourfold for galangin and eightfold for chrysin that also lacks an hydroxyl group at position 3 of ring C (Table 2). In both cases, prenylation by 3,3-DMA at position 8 also markedly increased (6- to 15-fold)



FIG. 2. Interaction of recombinant *Leishmania* NBD2 with silybin and derivatives monitored by the quenching of protein intrinsic fluorescence. (A) Spectral modification upon interaction with silybin. The fluorescence spectrum of 0.5 μ M NBD2 was recorded after excitation at 288 nm, in the absence (continuous line) or presence of either 4 μ M (broken line) or 16 μ M (dotted line) silybin; the traces were obtained by buffer subtraction before correction for inner-filter effect. (B) The concentration-dependent binding of silybin and derivatives was analyzed by quenching of NBD2 intrinsic fluorescence, determined by spectral integration from 300 to 350 nm, and corrected for the innerfilter effect in the presence of increasing concentrations of either silybin (\blacktriangle), DHS (\Box), or 8-(3,3-DMA)-DHS (\textcircled) added as a dimethyl sulfoxide solution.

the binding affinity. A double increase (12- to 28-fold) was even obtained with the 1,1-DMA substituent.

Sensitization of promastigote forms by silybin and its derivatives. The resistance to daunomycin in the MDR *L. tropica* line is mainly due to the overexpression of a Pgp-like transporter involved in drug efflux (4) which limits drug accumulation. We tested whether the binding of silybin derivatives to the cytosolic domain of the Pgp-like transporter could inhibit drug pumping and overcome drug resistance. Potential modulators were assessed for their ability to inhibit the growth of resistant parasites in the presence of daunomycin, in comparison with wild-type parasites in absence of drug. Figure 3 shows that a 72-h incubation of resistant parasites with 150 μ M daunomycin

TABLE 1. Effects of DHS prenylation on the affinity of binding to Leishmania NBD2^a

DHS derivative K_d (μ M ± SD)% Maximal quenching ± S6-Geranyl-DHS0.67 ± 0.0656.7 ± 1.48-Geranyl-DHS0.31 ± 0.0572.0 ± 2.26 (3.2 Dimothylelyl)DHS0.27 ± 0.0576.1 ± 2.4			
6-Geranyl-DHS 0.67 ± 0.06 56.7 ± 1.4 8-Geranyl-DHS 0.31 ± 0.05 72.0 ± 2.2 6 (3.2 Dimothylallyl) DHS 0.27 ± 0.05 76.1 ± 2.4	DHS derivative	$(\mu M \pm SD)$	$\%$ Maximal quenching \pm SD
$0-(3,3-Dimethylallyl)$ -DHS 0.27 ± 0.03 70.1 ± 2.4 $8-(3,3-Dimethylallyl)$ -DHS 0.11 ± 0.02 76.1 ± 1.7	6-Geranyl-DHS 8-Geranyl-DHS 6-(3,3-Dimethylallyl)-DHS 8-(3,3-Dimethylallyl)-DHS	$\begin{array}{c} 0.67 \pm 0.06 \\ 0.31 \pm 0.05 \\ 0.27 \pm 0.05 \\ 0.11 \pm 0.02 \end{array}$	$56.7 \pm 1.4 \\72.0 \pm 2.2 \\76.1 \pm 2.4 \\76.1 \pm 1.7$

^{*a*} The NBD2 domain was incubated, under the conditions of Fig. 2B, with the indicated hydrophobic derivatives of DHS. The dissociation constant and maximal quenching values were determined by fitting with the Graft program using the Erithacus software (see Materials and Methods).

in the presence of different silvbin derivatives gave differential dose-dependent growth inhibition (GI). These studies demonstrate the importance of silvbin oxidation to DHS and the prenylation of the latter. Consistent with the binding analysis, 8-(3,3-DMA)-DHS was the most efficient sensitizer. It gave more than 95% GI at 10 µM and showed only a minor toxic effect in the wild-type line. The other hydrophobically substituted derivatives also showed considerable sensitization of the cells (89 to 98% GI in the resistant line) at a threefold-higher concentration (30 µM). However, this concentration of geranyl derivatives gave significant cytotoxicity in the wild-type parasites (31 to 62% GI). In contrast, unmodified silvbin only gave modest sensitization, even at much higher concentrations (100 to 300 µM). Silymarin also did not reverse the resistant phenotype at high concentrations such as 250 µg/ml (data not shown). Finally, DHS showed considerable cytotoxicity on wild-type parasite (ca. 62% GI at 20 µM). This hampered studies of its sensitization of the MDR phenotype.

The reversal of parasite resistance to daunomycin was also tested with prenylated derivatives of galangin and chrysin. Figure 4A shows that higher concentrations of galangin derivatives were required to produce ca. 80% GI in the resistant line [20 μ M 8-(1,1-DMA)-galangin or 50 μ M 8-(3,3-DMA)-galangin] compared with 8-(3,3-DMA)-DHS (5 to 10 μ M), while nonprenylated galangin produced a slight sensitization at 75 μ M. The chrysin derivatives were even less efficient (Fig. 4B): 30 μ M 8-(1,1-DMA)-chrysin produced a 88% GI in the resistant line, and 40 μ M 8-(3,3-DMA)-chrysin gave 71% GI, but this concentration was relatively cytotoxic for the wild-type line. The nonsubstituted chrysin produced a small effect at 75 μ M. Interestingly, in both galangin and chrysin derivatives, the 1,1-DMA substitution gave better sensitivity to daunomycin that the 3,3-DMA substitution.

DISCUSSION

We show here that oxidized and prenylated derivatives of the therapeutic agent silybin exhibit high binding affinity to the recombinant cytosolic domain of *Leishmania* Pgp-like transporter and revert the MDR of an *L. tropica* line that overexpresses the transporter, drawing attention to the importance of the monolignol unit within the above compounds.

Silybin is a natural flavanolignan with many positive therapeutic properties and few adverse effects in animals and humans. Although its cellular target is unknown, it increased the cytotoxicity of doxorubicin in a doxorubicin-resistant cell line (36). In order to circumvent MDR phenotype in *Leishmania*,

TABLE 2. Binding affinities of different prenylated compounds lacking the monolignol unit^a-

Compound	$(\mu M \pm SD)$	% Maximal quenching ± SD	Source or reference
Galangin 8-(3,3-Dimethylallyl)-galangin 8-(1,1-Dimethylallyl)-galangin Chrysin 8-(3,3-Dimethylallyl)-chrysin 8-(1,1-Dimethylallyl)-chrysin	$\begin{array}{c} 9.2 \pm 1.0 \\ 0.62 \pm 0.24 \\ 0.34 \pm 0.09 \\ 17.6 \pm 5.9 \\ 2.9 \pm 0.3 \\ 1.4 \pm 0.2 \end{array}$	$75.9 \pm 3.9 \\ 80.0 \pm 6.4 \\ 85.0 \pm 4.0 \\ 81.3 \pm 8.6 \\ 84.3 \pm 2.0 \\ 75.4 \pm 2.7$	33 This study This study 33 This study 33

^{*a*} The NBD2 domain was incubated, under the conditions of Fig. 2B, with the indicated compounds, and the binding analyses were performed as described in Table 1.

we have studied silvbin binding to recombinant NBD2 of the L. tropica multidrug transporter, the effects of its oxidation to DHS, and the effects of different hydrophobic substitutions on ring A, previously described as critical for the increase the binding of flavones to the cytosolic domain of Leishmania Pgp-like transporter (33). These results, together with comparative data for derivatives lacking the monolignol unit, provide important structure-activity information. (i) First of all, these results demonstrate the significance of the oxidation of 2,3bond within the silybin ring C to its corresponding flavonol DHS, which may reinforce the mimicry of the adenine moiety of ATP, as previously suggested from the higher binding affinity of the flavone apigenin compared to its reduced analogue naringenin (7, 33). Conversely, the reduction of the 2,3-double bond of flavones to give flavanones resulted in a decrease of the competitive inhibition of H⁺,K⁺-ATPase with respect to ATP (29). (ii) They demonstrate the importance of the addition of a prenvl or geranyl hydrophobic substituent on ring A that could increase the interaction with the hydrophobic region vicinal to the ATP site (33). (iii) The monolignol unit (rings D and E in Fig. 1A and B) within flavanolignans produces significant effects with a five- to sixfold-higher affinity for 8-(3,3-DMA)-DHS with respect to 8-(3,3-DMA)-galangin and a fourfold higher affinity for DHS with respect to galangin. Additional studies are needed to determine its specific role in the interaction with the domain. (iv) These results further demonstrate the preference for hydrophobic substitution at position 8 over position 6 of ring A, suggesting some differences in binding orientation of the differently substituted compounds. (v) These results show the more efficient effect of prenylation compared to geranylation despite a lower hydrophobicity. (vi) Finally, these studies show the systematically more efficient effect of the 1,1-DMA compared to 3,3-DMA.

Similar in vitro results with some of these silybin derivatives have been obtained in parallel studies with the cytosolic domain of mammalian Pgp (26), except that the geranyl substitution was more efficient than the prenyl one. These differential results may indicate some differences between the cytosolic domains of *Leishmania* and mammalian transporters, possibly at the level of the hydrophobic interacting region.

The same sequence in efficiency has also been obtained from the in vitro sensitization studies in an MDR *Leishmania* line, so that compounds that display higher binding affinity for the recombinant NBD2 most efficiently sensitize the MDR phenotype. Thus, although the reversing effects of the compounds



FIG. 3. Sensitization by silybin derivatives in a daunomycin-resistant *L. tropica* line. Cell growth of either wild-type or resistant parasites was determined after incubation at 28°C for 72 h. Wild-type parasites (*) were incubated in the presence of different concentrations of silybin derivatives. Resistant parasites were incubated with the same concentrations of silybin derivatives in the presence of 150 μ M daunomycin. The results are expressed as the percentage of growth inhibition observed in each cell line compared to the absence of modulator (control cells). The data are means with standard deviations for three experiments performed in duplicate.

could in some cases be partially covered by their intrinsic cytotoxicity, as was observed for geranyl-DHS, the importance of the monolignol unit is evident from the higher reversion of resistance obtained with 8-(3,3-DMA)-DHS with respect to 8-(3.3-DMA)-galangin, as well as the role of prenvlation, especially 1,1-DMA, at position 8 of ring A. Thus, 8-(3,3-DMA)-DHS is the most active MDR-sensitizing agent ever described for Leishmania. Work is in progress to synthesize the 8-(1,1-DMA)-DHS derivative that would probably bind with even higher affinity to the domain and sensitize MDR at even lower concentrations. The single exception to the correlation between binding to NBD2 and MDR sensitization was the absence of any reversion by DHS. This appears to be due to the high cytotoxicity caused by this compound (ca. 62% GI in wild-type parasites at 20 µM) compared to silvbin (29% at 300 μM). Similarly, a fourfold-higher GI for DHS compared to that for silvbin has been described in a human ovarian carcinoma cell line (36). This effect could be due to higher mimicry with ATP after oxidation of the silybin 2,3-bond, thereby favoring additional binding to other ATP-binding proteins. Indeed, flavonols such as DHS, with a hydroxyl at position 3 and a 2,3-double bond in addition to the hydroxyl at position 5 and the ketone at position 4, contain all the requirements to bind to ATP-binding site, as previously shown not only for L. tropica (33) and mammalian (7, 11) multidrug transporters but also for crystallized CDK2 (10) and HcK (40).

We propose that prenylation of the ring A within these compounds might generate more specific inhibitors of Pgp-like transporters by strengthening the interaction with its cytosolic domains, possibly with the hydrophobic region vicinal to the ATP site. This would, on the one hand, increase the reversal efficiency on the MDR parasites and, on the other hand, lower the affinity for other cellular ATP-binding proteins, as deduced from the significant decrease of the cytotoxicity on wild-type parasites after prenylation (ca. 10% GI for 40 μ M 6-prenyl-DHS compared to nearly 100% GI for DHS at the same concentration; data not shown)

A number of observations have indicated flavonoid antagonism toward ATP: (i) the binding of kaempferide or derivatives to recombinant NBD2 from either mammalian (7) or *Leishmania* (33) multidrug transporter was partly prevented or displaced by ATP; (ii) flavonoid inhibition of several ATP-utilizing enzymes involves competitive interaction at the ATPbinding site (28), as studied in detail for H^+,K^+ -ATPase (29) and as clearly demonstrated by crystallization experiments with the protein kinases CDK2 (10) and Hck (40); and (iii) the flavonol quercetin, on the one hand, competitively inhibited the ATPase activity of a recombinant NBD2 from the cystic fibrosis transmembrane conductance regulator (another ABC protein) (35) and, on the other hand, prevented Hoechst 33342 transport by mammalian Pgp, partly by inhibiting its ATPase activity (38). We show here that the sensitization caused by



FIG. 4. Sensitization of growth caused by prenylated derivatives lacking the monolignol unit. Wild-type (*) and resistant parasites were incubated under the conditions of Fig. 3 with different prenylated derivatives of galangin (A) and chrysin (B). The data are means with standard deviations for three experiments performed in duplicate.

silybin derivatives in an MDR *L. tropica* line correlates with their affinity of binding to the cytosolic NBD2, which is consistent with a previous correlation between the binding affinity of other flavonoids for NBD2 and the increase of intracellular daunomycin accumulation monitored by flow cytometry (33). All of these results suggest that the reversion of drug resistance caused by silybin derivatives was produced by direct interaction with the nucleotide-binding domain of the Pgp-like transporter. However, other factors such as interaction with membrane phospholipids, altering the lipid packing density and the diffusion rate of the drug (6), or modulation of the multidrug transporter expression (22) may contribute to the observed sensitization of MDR. In addition, it is possible that prenylated derivatives might mainly interact at the hydrophobic region characterized within the NBDs or bind to the drug-binding sites within the transmembrane domains of the Pgp-like multidrug transporter. Quercetin has indeed been proposed to interact at the mammalian Pgp site where the transported drugs Hoechst 33342 and colchicine bind, which stimulated the transport of anthracyclines (38). Additional work is therefore required to further characterize the flavonoid-binding site(s).

In India, recent clinical studies showed that approximately 50% of patients with leishmaniasis fail to achieve parasite clearance after a standard dose of pentavalent antimonials (42). The in vivo data are supported by in vitro monitoring of drug sensitivity of fresh clinical isolates obtained at the same study site (24). The high rate of therapeutic failure to these usual drugs in cases of leishmaniasis call for new rational approaches to develop alternative drugs. Some pharmaceutical compounds, such as azoles and rifampin (27), and other drugs considered as potential leishmanicidal agents, such as doxorubicin (23), taxol (21) and alkyl lysophospholipids (44), are known substrates of Pgps or other ABC transporters and thus could induce an MDR phenotype. The development of inhibitors which block the MDR mechanism is a promising potential way to circumvent resistance to drugs. Our studies also provide useful models for understanding how similar defense mechanisms can be overcome in other protozoan parasites such as Plasmodium, Entamoeba, and Trichomonas spp., where ABC transporters have been associated with drug resistance.

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