Protein Kinase C Effectors Bind to Multidrug ABC Transporters and Inhibit Their Activity†

Gwenaëlle Conseil,‡ José Maria Perez-Victoria,§ Jean-Michel Jault,‡ Francisco Gamarro,§ André Goffeau,|| Johann Hofmann,⊥ and Attilio Di Pietro*,‡

Laboratoire des Protéines de Résistance aux Agents Chimiothérapeutiques, Institut de Biologie et Chimie des Protéines, UMR 5086 CNRS/Université Claude Bernard-Lyon I, Lyon, France, Instituto de Parasitologia y Biomedicina "Lopez-Neyra", Consejo Superior de Investigationes Cientificas, Granada, Spain, Unité de Biochimie Physiologique, Université Catholique de Louvain, Louvain-La-Neuve, Belgium, and Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Innsbruck, Austria

Received October 23, 2000; Revised Manuscript Received December 13, 2000

ABSTRACT: P-Glycoprotein and homologous multidrug transporters contain a phosphorylatable linker sequence that was proposed to control drug efflux on the basis that it was indeed phosphorylated in vitro and in vivo, and that inhibitors of protein kinase C (PKC) inhibited both P-glycoprotein phosphorylation and activity. However, site-directed mutagenesis of all phosphorylatable residues did not alter the drug resistance. The present work shows that PKC effectors are able to bind directly to multidrug transporters, from either cancer cells (mouse P-glycoprotein), yeast (*Saccharomyces cerevisiae* Pdr5p), or protozoan parasite (*Leishmania tropica* ltmdr1), and to inhibit their energy-dependent drug-efflux activity. The binding of staurosporine and derivatives such as CGP 41251 is prevented by preincubation with ATP, suggesting at least partial interaction at the ATP-binding site. In contrast, more hydrophobic compounds such as calphostin C and CGP 42700 bind outside the ATP-binding site and strongly interfere with drug interaction. A direct correlation is obtained between the efficiencies of PKC effectors to inhibit energy-dependent interaction of rhodamine 6G with yeast Pdr5p, to promote intracellular drug accumulation in various multidrug resistant cells, and to chemosensitize growth of resistant cells. The noncompetitive inhibition by PKC effectors of rhodamine 6G interaction with Pdr5p suggests that the binding might interfere with signal transduction between nucleotide hydrolysis and drug interaction. The overall results indicate that the multidrug transporters from different species display common features for interaction with PKC inhibitors. The hydrophobic derivative of staurosporine, CGP 42700, constitutes a potentially powerful modulator of P-glycoprotein-mediated multidrug resistance.

Multidrug resistance (MDR)[1] is a common cellular phenotype observed in all eukaryotic species. In mammalian cancer cells, it is often related to overexpression of P-glycoprotein (*1*, *2*), an ABC (ATP-binding cassette) multidrug transporter which confers resistance to anticancer drugs. Homologous transporters are found in yeasts such as *Saccharomyces cerevisiae* (*3*−*5*) and *Candida albicans* (*6*), conferring resistance to antifungals, as well as in protozoan parasites (*7*, *8*), such as *Plasmodium falciparum* (*9*, *10*), *Entamoeba* (*11*), and *Leishmania* (*12*, *13*), conferring resistance to antiparasitic drugs.

Despite some structural differences, all the transporters contain a central linker region that can be phosphorylated by protein kinases. P-Glycoprotein is indeed phosphorylated both in vitro and in vivo, especially by protein kinase C (PKC) (*14*−*17*). Well-known PKC inhibitors prevent PKC-mediated phosphorylation of P-glycoprotein, and induce intracellular drug accumulation and reversal of the P-glycoprotein-dependent MDR phenotype (*18*−*24*). Phosphorylation of P-glycoprotein was therefore proposed to play an important role in its drug-efflux activity, by analogy with another ABC transporter, CFTR (cystic fibrosis transmembrane conductance regulator), where phosphorylation of the

† This work was supported by CNRS/CGRI-FNRS agreements between A.D.P. and A.G. and CNRS/CSIC agreements between A.D.P. and F.G., by grants from the French Centre National de la Recherche Scientifique and Université Claude Bernard de Lyon (UMR 5086), the Association pour la Recherche sur le Cancer (ARC 9147), the Ligue Nationale contre le Cancer (Comités Départementaux du Rhône et de la Loire), the Belgian Service de la Politique Scientifique, Action Sciences de la Vie, and Fonds National de la Recherche Scientifique, and by Spanish Grant CICYT-FEDER (IFD97-0747-C04-03). G.C. was the recipient of fellowships from the Ligue Nationale contre le Cancer (Comité Départemental de la Haute-Savoie), the Région Rhône-Alpes (EURODOC Program), and the Fondation pour la Recherche Médicale. J.M.P.-V. was the recipient of a fellowship from the Spanish Project CICYT-FEDER.

* To whom correspondence should be addressed: Laboratoire des Protéines de Résistance aux Drogues Chimiothérapeutiques, Institut de Biologie et Chimie des Protéines, UMR 5086 CNRS-Université Claude Bernard-Lyon I, 7 Passage du Vercors, 69367 Lyon Cedex 07, France. Telephone: 33-47272 2629. Fax: 33-47272 2605. E-mail: a.dipietro@ibcp.fr.

‡ UMR 5086 CNRS/Université Claude Bernard-Lyon I.

§ Consejo Superior de Investigationes Cientificas.

|| Université Catholique de Louvain.

⊥ University of Innsbruck.

[1] Abbreviations: ABC, ATP-binding cassette; EDTA, ethylenediaminetetraacetic acid; HECAMEG, 6-*O*-(*N*-heptylcarbamoyl)methyl-α-D-glucopyranoside; MDR, multidrug resistance; NBD1, N-terminal nucleotide-binding domain; NBD2, C-terminal nucleotide-binding domain; PKC, protein kinase C; RU 486 (mifepristone), 17*β*-hydroxy-11*β*-[4-(dimethylaminophenyl)]-17α-(prop-1-ynyl)estra-4,9-diene-3-one.

10.1021/bi002453m CCC: $20.00
Published on Web 01/31/2001

central regulatory domain is critical for activity (*25*−*28*). However, site-directed mutagenesis of all phosphorylatable serine residues in the P-glycoprotein linker region produced no marked effect on drug efflux and the related MDR phenotype (*29*, *30*).

We recently prepared purified recombinant cytosolic nucleotide-binding domains of mouse P-glycoprotein (*31*−*34*) and *Leishmania tropica* ltrmdr1 (*35*) which were used to assess, by quenching of protein intrinsic fluorescence, the direct interaction with a number of substrates or modulators. The direct binding of effectors to purified full-length Pdr5p from *S. cerevisiae* could also be studied by the same fluorescence method (*36*).

The present work proves that PKC inhibitors, such as calphostin C, staurosporine, and hydrophobic derivatives are in fact able to bind directly to the different purified multidrug transporter or cytosolic domains. The binding of staurosporine and its CGP 41251 derivative partly overlapped the ATP-binding site, whereas the more hydrophobic CGP 42700 bound outside and strongly inhibited energy-dependent drug interaction within yeast Pdr5p, as well as nucleotide hydrolysis to a lower extent. Due to its lower intrinsic cytotoxicity, CGP 42700 binding was able to induce a differential reversal of the MDR phenotype in a daunomycin-resistant *L. tropica* line.

EXPERIMENTAL PROCEDURES

Materials. The PKC inhibitors bisindolylmaleimide I, Gö 6976, H-7, H-89, hypericin, K252a, and pseudohypericin were purchased from Calbiochem Novabiochem. Calphostin C, mezerein, and staurosporine came from Alexis Biochemicals. ATP was from Roche Molecular Biochemicals, UTP from Sigma, and rhodamine 6G from Merck. RU 486 was obtained as described previously (*33*), and CGP 41251 and CGP 42700 were kindly provided by Novartis Pharma AG (Basel, Switzerland).

Biological Preparations. Recently published procedures were used to prepare recombinant NBD2 from either mouse P-glycoprotein (*34*) or the *L. tropica* multidrug transporter (*35*), as well as Pdr5p-enriched plasma membranes and the purified Pdr5p transporter from *S. cerevisiae* mutant strain AD124567 (*36*). Wild-type and daunomycin-resistant *L. tropica* lines were used as described previously (*35*).

Assay Procedures. The quenching of protein intrinsic fluorescence, due to direct interaction with PKC effectors, was studied as previously described for flavonoids with recombinant NBD2 from either mouse P-glycoprotein (*34*) or the *L. tropica* multidrug transporter (*35*), or with the purified yeast Pdr5p transporter (*36*). UTPase activity and energy-dependent quenching of rhodamine 6G fluorescence were assayed as described previously (*36*). Parasite culture and in vivo chemosensitization of growth to daunomycin were studied as described previously (*35*).

RESULTS

Direct Interaction of PKC Inhibitors with a Cytosolic Domain of P-Glycoprotein. The potent PKC inhibitor staurosporine, whose chemical structure is drawn in Figure 1, interacted directly with recombinant NBD2 as monitored by quenching of the domain intrinsic fluorescence (Figure 2). Analysis of the data with the Grafit program gave a maximal quenching value ΔF_{max} of 25.7 ± 2.1% and a K_D of 1.78 ± 0.53 μM. The level of binding strongly decreased by preincubating NBD2 with 10 mM ATP regardless of whether Mg^{2+} ions were present. A higher maximal quenching of 55.6 ± 4.6% was produced by CGP 41251, a hydrophobic staurosporine derivative in which the pyran heterocycle is substituted with a benzoyl function (cf. Figure 1). CGP 41251 binding was as well partly prevented by preincubation with 10 mM ATP, but to a lesser extent than staurosporine (not shown here). The even more hydrophobic derivative CGP 42700, where the amide function is additionally substituted with a benzyl group, displayed a biphasic binding curve, with a first phase corresponding to high affinity (maximal quenching of ∼15−20% and a sub-micromolar K_D value) followed by a lower-affinity phase leading to complete maximal quenching with a half-maximal effect at ∼12 μM.

Figure 3 shows that the other well-known PKC inhibitor calphostin C (cf. structure in Figure 1) was also able to bind to NBD2. Maximal quenching (ΔF_{max} = 38.0 ± 2.3%) and binding affinity (K_D = 0.30 ± 0.13 μM) were even higher than for staurosporine, but the interaction was not altered by preincubation with ATP (Figure 3). In contrast, calphostin C efficiently antagonized RU 486 binding to NBD2 (inset), which was previously shown to involve a hydrophobic steroid-interacting region close to the ATP-binding site (*33*, *34*). CGP 41251 also altered RU 486 interaction, but to a lesser extent than calphostin C (data not shown).

Direct Interaction of PKC Inhibitors with Yeast Pdr5p and Inhibition of both the UTPase Activity and Energy-Dependent Interaction with Rhodamine 6G. Both staurosporine and calphostin C also bound to purified Pdr5p (Figure 4), producing a higher maximal quenching of intrinsic fluorescence (45 ± 6.9 and 83.7 ± 0.94%, respectively) than for P-glycoprotein NBD2, while the K_D values were slightly higher (3.1 ± 1.1 and 0.82 ± 0.05 μM, respectively). Like that of P-glycoprotein NBD2, the interaction of Pdr5p with staurosporine was prevented by ATP, whereas calphostin C binding was ATP insensitive.

Both staurosporine and calphostin C inhibited the UTP hydrolysis catalyzed by Pdr5p-enriched plasma membranes, with a much higher affinity for the latter compound since levels of inhibition of initial activity of 12 and 50%, respectively, were observed at a concentration of 10 μM (Figure 5). The hydrophobic derivatives of staurosporine, CGP 41251 and CGP 42700, behaved as much more potent inhibitors, since a 50% inhibition was observed at ∼4 and ∼2 μM, respectively. Like that of calphostin C, the inhibition of UTPase activity was partial and did not exceed 55−60%. The approximate concentrations producing a half-maximal effect were 4, 1, and 0.5 μM for calphostin C, CGP 41251, and CGP 42700, respectively.

Staurosporine and its hydrophobic derivatives produced a more potent inhibition on the energy-dependent interaction of rhodamine 6G with the same yeast plasma membranes (Figure 6A). The inhibition of the quenching of 144 nM rhodamine 6G fluorescence by 4.8 mM MgATP reached completion in all cases, with $I_{50\%}$ values of 1.1 ± 0.09, 0.16 ± 0.04, and 0.066 ± 0.004 μM for staurosporine, CGP 41251, and CGP 42700, respectively. In contrast, no quenching was observed in the absence of magnesium ions or in the presence of either MgADP (*37*) or MgAMP (data not shown). Neither staurosporine nor its derivatives modified

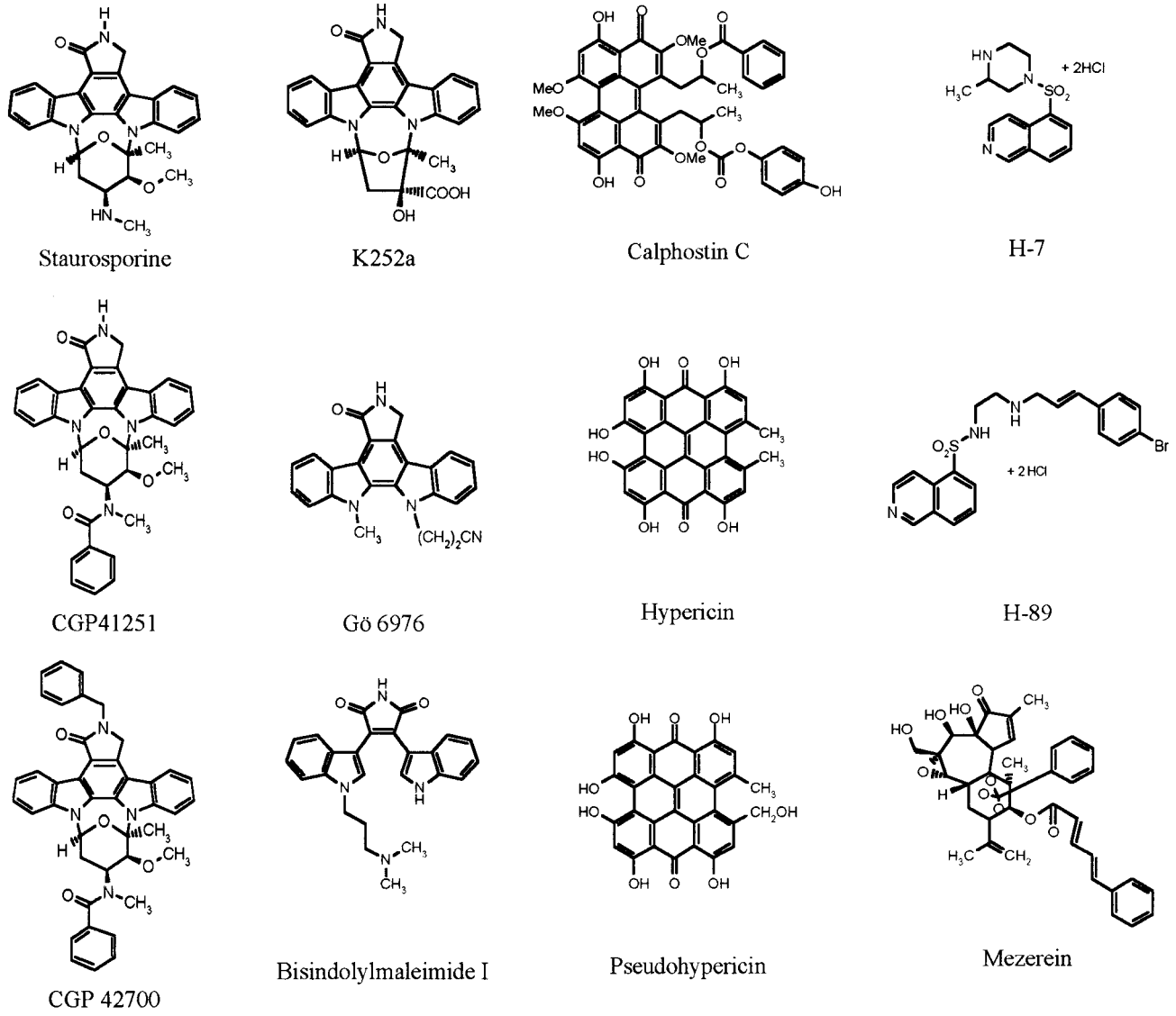

FIGURE 1: Chemical structures of the PKC effectors that have been studied. The two left columns show staurosporine and different derivatives. The two right columns reassemble calphostin C and analogues together with various other, inhibitory or activating, effectors.

rhodamine 6G fluorescence in the absence of MgATP. The type of inhibition was studied at increasing rhodamine 6G concentrations as illustrated here for CGP 42700 (Figure 6A inset). The extent of inhibition produced by increasing CGP 42700 concentrations, up to 0.2 μM, appeared to be independent of the rhodamine 6G concentration, which was indicative of noncompetitive inhibition. A similar type of inhibition was also observed for the following PKC effectors: CGP 41251, staurosporine, and mezerein (data not shown). A wide array of inhibition efficiencies was obtained when using a series of PKC effectors (cf. stuctures in Figure 1) as illustrated in Figure 6B. The following order of inhibitory efficiency was observed: mezerein > Gö 6976 > H-89 > pseudohypericin > K252a > bisindolylmameimide I > hypericin > H-7. The corresponding $I_{50\%}$ values are collected in Table 1; it is worth mentioning that the PKC activator, mezerein, is an inhibitor with a potency similar to that of the hydrophobic derivative of staurosporine, CGP 42700.

Direct Binding of PKC Effectors to the Cytosolic Domain of the L. tropica Multidrug Transporter and Chemosensitization of Parasite Growth to Daunomycin. Staurosporine and calphostin C also bound to recombinant NBD2 of the parasite multidrug transporter (Figure 7). As for P-glycoprotein NBD2 and full-length Pdr5p, calphostin C produced both a higher level of maximal quenching ($\Delta F_{max} = 70.9 \pm 2.6\%$) and a higher affinity ($K_D = 0.73 \pm 0.14$ μM) than did staurosporine, with values of 42.6 $\pm$ 1.4% and 7.7 $\pm$ 0.9 μM, respectively.

Both hydrophobic derivatives of staurosporine were able to abolish the resistance of *L. tropica* lines to daunomycin (Figure 8). CGP 41251 fully abolished the growth of the resistant line in the presence of daunomycin at low micromolar concentrations, but a similar effect was observed with the parental line in the absence of drug. In contrast, a differential effect was produced by CGP 42700, since the growth of the resistant line was considerably more inhibited than that of the wild-type line (46.2 $\pm$ 4.6 vs 5.0 $\pm$ 1.0%) in the presence of 100 μM CGP 42700.

DISCUSSION

The main new results of this paper concern the direct binding of PKC effectors to P-glycoprotein and related

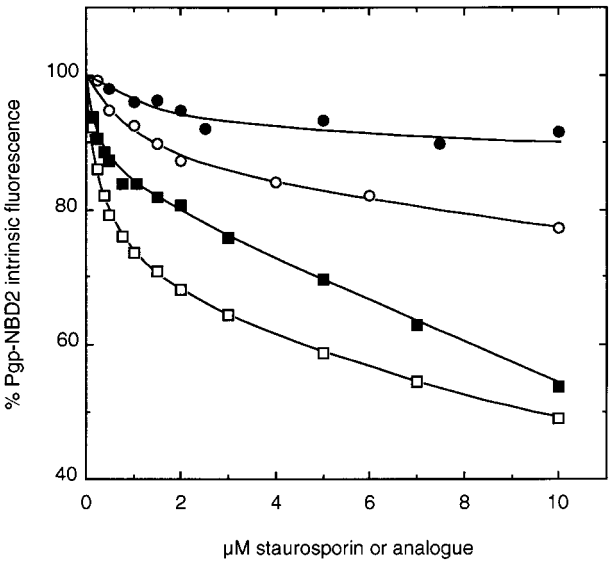

FIGURE 2: Direct interaction of staurosporine and hydrophobic analogues with the C-terminal cytosolic nucleotide-binding domain of P-glycoprotein. The purified recombinant cytosolic domain of mouse P-glycoprotein, NBD2 [at 0.5 μM in 20 mM potassium phosphate, 0.5 M NaCl, 20% glycerol, 0.01% HECAMEG, and 5 mM β-mercaptoethanol (pH 6.8)], was incubated at 25.0 $\pm$ 0.1 °C with the indicated concentrations of either staurosporine (○) or its hydrophobic derivatives, CGP41251 (□) or CGP42700 (■). When indicated, the domain was preincubated for 20 min with 10 mM ATP before interaction with staurosporine (●). The excitation wavelength was set at 295 nm and the fluorescence emission recorded from 310 to 360 nm and integrated. Measurements were corrected for buffer contribution and inner filter effects of the compounds measured in parallel experiments with *N*-acetyltryptophanamide, and analyzed with the Grafit program.

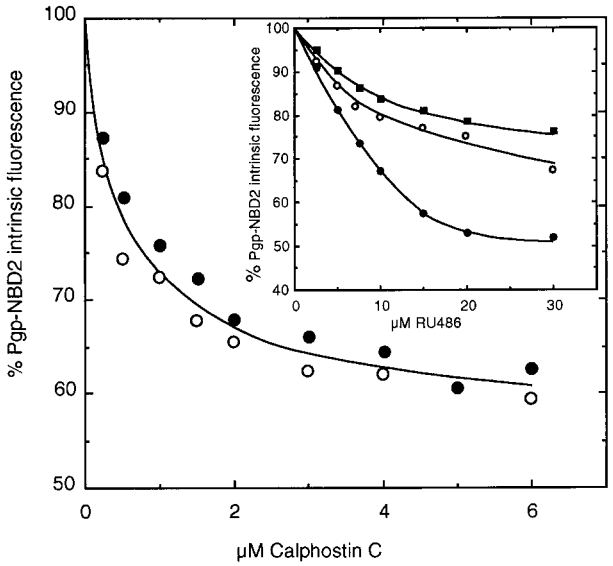

FIGURE 3: Binding of calphostin C and antagonism with RU 486. The quenching of NBD2 intrinsic fluorescence induced upon interaction with calphostin C was studied under the same experimental conditions as described in the legend of Figure 2, after preincubation in the absence (●) or presence (○) of 10 mM ATP. (Inset) The binding of RU486 was assessed after preincubation of the protein for 20 min in the absence (●) or presence of calphostin C at 0.5 (○) or 2 μM (■).

multidrug ABC transporters. This binding is responsible for inhibition of drug interaction, inhibition of nucleoside triphosphate hydrolysis, and reversal of cell multidrug resistance.

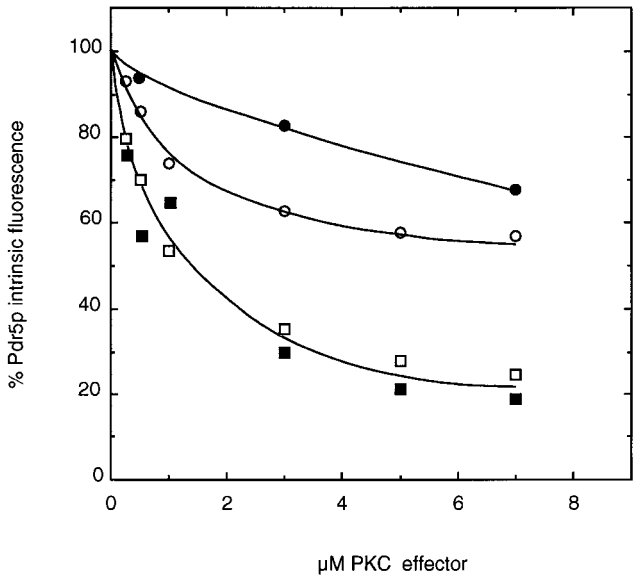

FIGURE 4: Direct interaction of PKC inhibitors with the purified Pdr5p multidrug transporter. Purified Pdr5p from *S. cerevisiae* was diluted to 0.05 μM (1 μM tryptophan residue) in 10 mM Tris-HCl, 12% (w/v) sucrose, 0.02% (w/v) *n*-dodecyl β-D-maltoside, and 5 mM $MgCl_2$ (pH 7.5), with staurosporine (circles) or calphostin C (squares), and preincubated (black symbols) or not (white symbols) with 10 mM ATP. Fluorescence conditions and corrections and analysis of the data were performed as described in the legend of Figure 2.

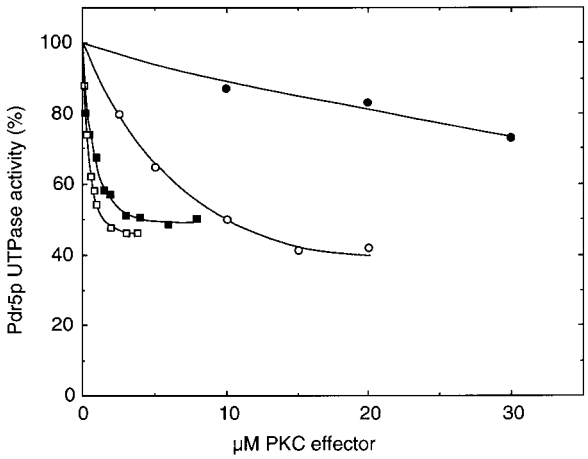

FIGURE 5: Inhibition of Pdr5p UTPase activity. Pdr5p-enriched membranes (10−20 μg of protein) were incubated at 35 °C in 500 μL of 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid], at pH 7.5, containing 0.3 mM ammonium heptamolybdate, 75 mM potassium nitrate, and 7.5 mM sodium azide, in the presence of increasing concentrations of either staurosporine (●), calphostin C (○), CGP 412151 (■), or CGP 42700 (□). The reaction was started by the addition of 6 mM UTP and excess $MgCl_2$ providing 1 mM free Mg^{2+}, and the phosphate that was released for 5−15 min was titrated colorimetrically.

The use of purified proteins and in vitro fluorescence assays has allowed here the demonstration of direct binding of the two well-known PKC inhibitors, staurosporine and calphostin C, to three multidrug transporters of various origins, namely, mouse P-glycoprotein, yeast Pdr5p, and protozoan parasite ltmdr1, suggesting a ubiquitous phenomenon. Staurosporine appears to bind, at least partly, to the ATP-binding site since a strong prevention of binding was provided by preincubation with ATP when using P-glycoprotein recombinant NBD2 or full-length Pdr5p. The ATP

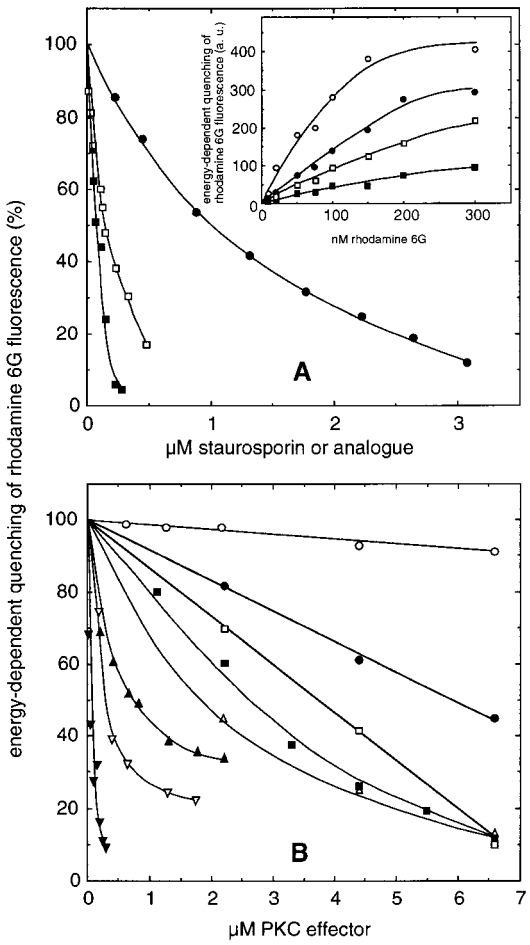

FIGURE 6: Inhibition of MgATP-dependent rhodamine 6G interaction with Pdr5p. (A) Inhibition by staurosporine and hydrophobic derivatives. Pdr5p-enriched plasma membranes (70−130 μg of proteins) were incubated at 30 °C in 2.2 mL of 50 mM Hepes (pH 7.0) in the presence of 144 nM rhodamine 6G and increasing concentrations of either staurosporine (●), CGP 412151 (□), or CGP 42700 (■). The reaction was initiated by addition of 4.8 mM $MgCl_2$ and ATP, and followed for 20−40 min by the decrease in fluorescence emission at 552 nm upon excitation at 525 nm. The $I_{50\%}$ values of rhodamine 6G fluorescence quenching were determined with the Grafit program as previously described (*37*). The inset of panel A shows the type of inhibition produced by CGP 42700; the membranes were incubated in the presence of increasing rhodamine 6G concentrations, as indicated, and CGP 42 700 at either 0 (○), 0.05 (●), 0.10 (□), or 0.20 μM (■), before MgATP addition. (B) Effects produced under the same conditions as described for panel A by increasing concentrations of the following other PKC effectors: H-7 (○), hypericin (●), bisindolylmaleimide I (□), K252a (■), pseudohypericin (△), H-89 (▲), Gö 6976 (▽), and mezerein (▼).

prevention was observed as well when ATP binding was independent of Mg^{2+} ions, for recombinant NBD2, as when ATP binding was Mg^{2+}-dependent, for Pdr5p (*36*). Since 8-azido-ATP was recently reported to interact differently with recombinant NBD1 of varying length regardless of whether binding was dependent on Mg^{2+} (*38*), our results suggest that the presence of Mg^{2+} might increase the affinity at the level of the nucleotide phosphate chain, but not change the type of interaction involving the adenosine moiety. This is consistent with both the crystallographic demonstration in cyclin-dependent kinase 2 (*39*) that staurosporine binding overlaps the adenosine moiety of the ATP-binding site and the lack of staurosporine prevention against azidopin photolabeling in P-glycoprotein-containing membranes (*22*). In contrast, calphostin C binds outside the ATP-binding site, since its interaction is not altered upon preincubation with ATP. The antagonism against RU 486 interaction strongly suggests that calphostin C interaction occurs within the hydrophobic region previously shown to bind steroids in both cytosolic domains of P-glycoprotein (*33*, *34*) and the *L. tropica* multidrug transporter (*35*).

The hydrophobic staurosporine derivative CGP 41251 displays a partial antagonism against both ATP and RU 486, indicating that its binding might partly overlap both the ATP-binding site and the steroid-interacting hydrophobic region. This is consistent with the fact that the pyran heterocycle is located outside the ATP-binding site in the cyclin-dependent kinase 2 structure (*39*), and its benzoylation is therefore expected not to prevent ATP interaction while strengthening possible interaction at the vicinal hydrophobic region. In contrast, the even more hydrophobic, disubstituted, derivative CGP 42700 appears to bind differently to P-glycoprotein NBD2 as evidenced from the biphasic curve of binding. This might be due to some shift toward the outside of the ATP-binding site since the amide function is indeed interacting through a hydrogen bond with Glu-81 in the cyclin-dependent kinase 2 structure (*39*); its substitution with a huge hydrophobic benzyl group might therefore induce a marked reorientation of the compound binding, possibly in better proximity to the steroid-interacting region. Furthermore, CGP 41251, but not CGP 42700, has been shown to mimic staurosporine in affecting many cellular pathways (*40*−*47*).

The PKC effectors that bind to multidrug transporters inhibit their activity with varying potencies. A complete inhibition was produced at micromolar or sub-micromolar concentrations on the energy-dependent drug interaction with Pdr5p as monitored by quenching of rhodamine 6G fluorescence. The potency was dependent on hydrophobicity as evidenced in the staurosporine series: CGP 42700 > CGP 41251 > Gö 6976 > staurosporine > K252a > bisindolylmaleimide I (Table 1). This was also the case for the adenine analogues: H-89 > H-7. In contrast, the fact that pseudohypericin is slightly less hydrophobic than hypericin indicates that other parameters also need to be taken into account. The different $I_{50\%}$ values obtained against rhodamine 6G interaction are consistent with the concentration range used with MDR cancer cell lines to observe drug intracellular accumulation and chemosensitization of cell growth. For example, the less efficient H-7, which here hardly inhibits rhodamine interaction at 10 μM, required a concentration as high as 100 μM for chemosensitization (*22*), while the most efficient CGP 42700 with a $I_{50\%}$ of 0.066 ± 0.004 μM was used at only 0.2 μM for reversal of the MDR phenotype (*24*). Other bisindolylmaleimides (RO 31-8220 and GF 109203X) were also found to be less efficient than staurosporine toward both intracellular drug accumulation and reversal of MDR phenotype in MCF-7/Adr cells (*23*). With regard to calphostin C, although its $I_{50\%}$ could not be determined due to strong interference with rhodamine 6G fluorescence, its efficiency might be expected to be at least 5-fold higher than that of staurosporine on the basis of its higher-affinity binding to all multidrug transporters; this is also consistent with the concentrations of 0.25−2 μM used to revert the MDR phenotype (*22*), whereas its K_i for PKC inhibition is 0.05 μM (*59*).

Table 1: Comparison between the Efficiency of PKC Effectors in Inhibiting Energy-Dependent Interaction of Pdr5p with Rhodamine 6G in This Work and Their Reported Inhibition of PKC Activity and Effects on MDR Cancer Cells

PKC effector	$I_{50\%}$ (rhodamine 6G interaction) (μM)	$I_{50\%}$ or K_i (PKC) (μM)	concentration required for cellular effects (μM)
H-7	low level of inhibition at 10 μM	6.0 (*48*)	100 (*22*)
hypericin	5.9 ± 0.1	3.4 (*49*)	nd[a]
bisindolylmaleimide I	4.5 ± 0.3	0.01 (*50*)	nd[a]
K252a	2.7 ± 0.09	0.025 (*51*)	1−8 (*22*)
pseudohypericin	1.6 ± 0.2	28.7 (*52*)	nd[a]
staurosporine	1.1 ± 0.09	0.0007 (*53*)	5[b] (*22*)
H-89	0.76 ± 0.19	31.7 (*54*)	5−10 (*22*)
Gö 6976	0.35 ± 0.09	6.2 (*55*)	nd[a]
CGP 41251	0.16 ± 0.04	0.05 (*56*, *57*)	0.15−0.20 (*24*, *57*)
mezerein	0.071 ± 0.005	activation (*58*)	0.5−5 (*22*)
CGP 42700	0.066 ± 0.004	>100 (*24*, *56*)	0.2 (*24*)

[a] nd, not determined. [b] Staurosporine could not be assayed for growth sensitization due to high intrinsic cytotoxicity (*22*).

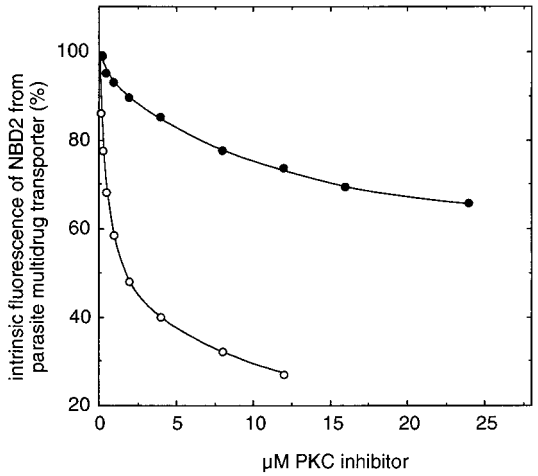

FIGURE 7: Binding of PKC inhibitors to the recombinant cytosolic domain of the *L. tropica* multidrug transporter. The intrinsic fluorescence of purified recombinant NBD2 of the *L. tropica* multidrug transporter (at 0.5 μM) in 1.2 mL of 50 mM potassium phosphate (pH 8.5), 1 M NaCl, 20% (w/v) glycerol, 0.05% (w/v) HECAMEG, 1 mM β-mercaptoethaol, and 10 mM imidazole was recorded in the range of 300−350 nm upon excitation at 288 nm. The binding of either staurosporine (●) or calphostin C (○) was analyzed as described in the legend of Figure 2.

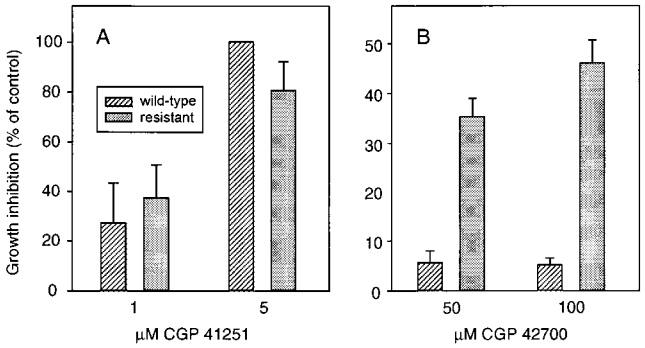

FIGURE 8: In vivo sensitization by staurosporine hydrophobic derivatives in a daunomycin-resistant *L. tropica* line. The extent of cell growth of either wild-type (without daunomycin) or resistant parasites (with 150 μM daunomycin) was determined after incubation at 28 °C for 48 h, in the presence of the indicated concentrations of either CGP 41251 or CGP 42700. The results are expressed as the percentage of growth inhibition by comparison with control cells grown in the absence of the staurosporine derivative. The means ± standard deviation of duplicate measurements of three independent experiments are shown.

In contrast, no correlation at all could be observed between the potency of compounds to inhibit the Pdr5p transporter activity and their efficiency to inhibit PKC activity (Table 1). A similar conclusion was proposed by Smith and Zilfou (*22*) from the effects of some PKC effectors on MDR cancer cells, concerning drug intracellular accumulation and cell growth chemosensitization. We further confirm here the lack of correlation for other compounds such as CGP 41251, CGP 42700, Gö 6976, bisindolylmaleimide I, and pseudohypericin and hypericin. It appears quite evident that the effects are correlated to direct binding to the multidrug transporter and not to inhibition of any phosphorylation, since the maximal effects were produced by CGP 42700, a very low level inhibitor (10^5-fold less efficient than staurosporine), and mezerein which is an activator of PKC.

These inhibitors seem not to bind to the drug-binding site(s) of Pdr5p since the inhibition is noncompetitive with respect to rhodamine 6G, as observed for CGP 42700 and all the other PKC effectors that have been tested. This correlates with the lack of marked prevention by staurosporine and mezerein against azidopin photolabeling (*22*). The inhibition of UTP hydrolysis is only partial, as it usually is limited to 55−60%, but follows the same order of efficiency as the inhibition of rhodamine 6G interaction: CGP 42700 > CGP 41251 > calphostin C > staurosporine. This indicates that the same molecule(s) of PKC effector bound to Pdr5p might be responsible for inhibition of both UTP hydrolysis and rhodamine 6G interaction. However, the latter is much more strongly inhibited than the former, since the required concentrations are 15−30-fold lower. The difference would be even higher with ATPase activity which has been shown previously to be generally 5−10-fold less sensitive to inhibitors than UTPase activity (*36*). Therefore, the binding of PKC effectors to Pdr5p appears to be around 200-fold more efficient in inhibiting drug interaction than ATP hydrolysis. The binding site is, at least partly, different from that of prenylflavonoids, other high-affinity inhibitors recently shown to bind to the drug-binding site(s) by producing competitive inhibition (*36*). A possibility for PKC effectors, especially the more hydrophobic ones such as CGP 42700 and mezerein, would be to bind outside both ATP- and drug-binding sites and to alter conformational change(s) related to signal transduction from nucleotide hydrolysis to drug interaction, maybe by interacting with a putative connecting transduction sequence as recently described in ArsA structure (*60*). One can wonder whether such an interacting site might correspond to one of the multiple modulator-binding sites identified within multidrug transporters (*61*−*63*).

Some more hydrophilic effectors such as staurosporine and, to a lesser extent, CGP 41251 might however partly overlap the ATP-binding site, as deduced from both the protection by ATP against their direct binding and the high intrinsic cytotoxicity of CGP 41251 in parasite cell lines. A very high intrinsic cytotoxicity was similarly observed with staurosporine in human MDR cells, likely due to interaction with PKC and other ATP-binding cellular targets, which prevented any differential study of cell growth chemosensitization (*22*). Conversely, CGP 42700, which is no longer able to bind to the ATP site due to hydrophobic substitution of the amide function, displays a very low intrinsic cytotoxicity on parasite cells and is indeed able to differentially chemosensitize the proliferation of the daunomycin-resistant *L. tropica* line. The efficient concentrations are similar to those required for conventional modulators such as verapamil and cyclosporin A, which are rather high due to the very high level of resistance of this parasite line kept in the presence of 150 μM daunomycin (*35*). Also in human KB and HeLa human cells, CGP 42700 was much less cytotoxic than CGP 41251 (*24*).

In conclusion, many PKC effectors are able to directly bind to P-glycoprotein and homologous multidrug transporters, and to inhibit their energy-dependent transport activity. Therefore, the previous use of PKC inhibitors to demonstrate the involvement of PKC-mediated activation of P-glycoprotein drug-transport activity must have overlooked this possibility (*18*−*21*). The overall results also indicate that the multidrug transporters from different species such as mammals, yeast, and protozoan parasites display common features for interaction with protein kinase effectors. The hydrophobic CGP 42700 appears to be particularly interesting as a potential modulator of the MDR phenotype since it strongly inhibits the multidrug transporter functioning without interfering with either PKC activity or cell growth.

ACKNOWLEDGMENT

We thank Novartis Pharma A. G. for kindly providing CGP 41251 and CGP 42700.

REFERENCES

1. Endicott, J. A., and Ling, V. (1989) *Annu. Rev. Biochem. 58*, 137−171.
2. Gottesman, M. M., and Pastan, I. (1993) *Annu. Rev. Biochem. 62*, 385−427.
3. Balzi, E., Wang, M., Leterme, S., Van Dyck, L., and Goffeau, A. (1994) *J. Biol. Chem. 269*, 2206−2214.
4. Decottignies, A., Kolaczkowski, M., Balzi, E., and Goffeau, A. (1994) *J. Biol. Chem. 269*, 12797−12803.
5. Decottignies, A., and Goffeau, A. (1997) *Nat. Genet. 15*, 137−145.
6. Prasad, R., De Wergifosse, P., Goffeau, A., and Balzi, E. (1995) *Curr. Genet. 27*, 320−329.
7. Ullman, B. (1995) *J. Bioenerg. Biomembr. 27*, 77−84.
8. Borst, P., and Ouellette, M. (1995) *Annu. Rev. Microbiol. 49*, 427−460.
9. Wilson, C. M., Serrano, A. E., Walsley, A., Bogenschutz, M. P., Sankar, A. H., and Wirth, D. F. (1989) *Science 244*, 1184−1186.
10. Foote, S. J., Thompson, J. K., Cowman, A. F., and Kemp, D. J. (1989) *Cell 57*, 921−930.
11. Samuelson, J., Ayala, P., Orozco, E., and Wirth, D. (1990) *Mol. Biochem. Parasitol. 38*, 281−290.
12. Hendrickson, N., Sifri, C. D., Allen, T., Wirth, D. F., and Ullman, B. (1993) *Mol. Biochem. Parasitol. 60*, 53−64.
13. Chiquero, M. J., Pérez-Victoria, J. M., O'Valle, F., Gonzales-Ros, J. M., del Moral, R., Ferragut, J. A., Castanys, S., and Gamarro, F. (1998) *Biochem. Pharmacol. 55*, 131−139.
14. Orr, G. A., Han, E. K.-H., Browne, P. C., Nieves, E., O'Connor, B. M., Yang, C.-P. H., and Bates, S. B. (1993) *J. Biol. Chem. 268*, 25054−25062.
15. Chambers, T. C., Pohl, J., Raynor, R. L., and Kuo, J. F. (1993) *J. Biol. Chem. 268*, 4592−4595.
16. Chambers, T. C., Pohl, J., Glass, D. B., and Kuo, J. F. (1994) *Biochem. J. 299*, 309−315.
17. German, U. A., Chambers, T. C., Ambudkar, S. V., Pastan, I., and Gottesman, M. M. (1995) *J. Bioenerg. Biomembr. 27*, 53−61.
18. Sato, W., Yusa, K., Naito, M., and Tsuruo, T. (1990) *Biochem. Biophys. Res. Commun. 173*, 1252−1257.
19. Ma, L., Marquardt, D., Takemoto, L., and Center, M. S. (1991) *J. Biol. Chem. 266*, 5593−5599.
20. Bates, S. E., Lee, J. S., Dickstein, B., Spolyar, M., and Fojo, A. T. (1993) *Biochemistry 32*, 9156−9164.
21. Gupta, K. P., Ward, N. E., Gravitt, K. R., Bergman, P. J., and O'Brian, C. A. (1996) *J. Biol. Chem. 271*, 2102−2111.
22. Smith, C. D., and Zilfou, J. T. (1995) *J. Biol. Chem. 270*, 28145−28152.
23. Budworth, J., Davies, R., Malkhandi, J., Gant, T. W., Ferry, D. R., and Gescher, A. (1996) *Br. J. Cancer 73*, 1063−1068.
24. Utz, I., Spitaler, M., Rybczynska, M., Ludescher, C., Hilbe, W., Regenass, U., Grunicke, H., and Hofmann, J. (1998) *Int. J. Cancer 77*, 64−69.
25. Berger, H. A., Travis, S. M., and Welsh, M. J. (1993*) J. Biol. Chem. 268*, 2037−2047.
26. Chang, X.-B., Tabcharani, J. A., Hou, Y.-X., Jensen, T. J., Kartner, N., Alon, N. A., Hanrahan, J. W., and Riordan, J. R. (1993*) J. Biol. Chem. 268*, 11304−11311.
27. Gadsby, D. C., and Nairn, A. C. (1994) *Trends Biochem. Sci. 19*, 513−518.
28. Ma, J., Zhao, J., Drumm, M. L., Xie, J., and Davies, B. (1997)- *J. Biol. Chem. 272*, 28133−28141.
29. Germann, U. A., Chambers, T. C., Ambudkar, S. V., Licht, T., Cardelli, C. O., Pastan, I., and Gottesman, M. M. (1996) *J. Biol. Chem. 271*, 1708−1716.
30. Goodfellow, H. R., Sardini, A., Ruetz, S., Callaghan, R., Gros, P., McNaughton, P. A., and Higgins, C. F. (1996) *J. Biol. Chem. 271*, 13668−13674.
31. Baubichon-Cortay, H., Baggetto, L. G., Dayan, G., and Di Pietro, A. (1994) *J. Biol. Chem. 269*, 22983−22989.
32. Dayan, G., Jault, J.-M., Baubichon-Cortay, H., Jault, J.-M., Cortay, J.-C., Deléage, G., and Di Pietro, A. (1996) *J. Biol. Chem. 271*, 11652−11658.
33. Dayan, G., Jault, J.-M., Baubichon-Cortay, H., Baggetto, L. G., Renoir, J. M., Baulieu, E. E., Gros, P., and Di Pietro, A. (1997) *Biochemistry 36*, 15208−15215.
34. Conseil, G., Baubichon-Cortay, H., Dayan, G., Jault, J.-M., Barron, D., and Di Pietro, A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9831−9836.
35. Pérez-Victoria, J. M., Chiquero, M. J., Conseil, G., Dayan, G., Di Pietro, A., Barron, D., Castanys, S., and Gamarro, F. (1999) *Biochemistry 38*, 1736−1743.
36. Conseil, G., Decottignies, A., Jault, J.-M., Comte, G., Barron, D., Goffeau, A., and Di Pietro, A. (2000) *Biochemistry 39*, 6910−6917.
37. Kolaczkowski, M., van der Rest, M., Cybularz-Kolaczkowska, A., Soumillon, J.-P., Konings, W. N., and Goffeau, A. (1996) *J. Biol. Chem. 271*, 31543−31548.
38. Booth, C. L., Pulaski, L., Gottesman, M. M., and Pastan, I. (2000) *Biochemistry 39*, 5518−5526.
39. Lawrie, A. M., Noble, M. E. M., Tunnah, P., Brown, N. R., Johnson, L. N., and Endicott, J. A. (1997) *Nat. Struct. Biol. 4*, 796−801.
40. Jelkmann, W., Huwiler, A., Fandrey, J., and Pfeilschifter, J. (1991) *Biochem. Biophys. Res. Commun. 179*, 1441−1448.
41. Chung, D. L., Brandt-Rauf, P. W., Weinstein, I. B., Nishimura, S., Yamaizumi, Z., Murphy, R. B., and Pincus, M. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1993−1996.

42. Susa, M., Vulevic, D., Lane, H. A., and Thomas, G. (1992) *J. Biol. Chem. 267*, 6905−6909.
43. Grosman, N. (1992) *Immunopharmacology 24*, 47−55.
44. Zimmermann, A., and Keller, H. (1992) *Br. J. Cancer 66*, 1077−1082.
45. Alkan, S. S., Rutschmann, S., Grogg, D., and Erb, P. (1993) *Cell. Immunol. 150*, 137−148.
46. Loneragan, K., Cheah, T. B., Bunn, S. J., and Marley, P. D. (1996) *Eur. J. Pharmacol. 311*, 87−94.
47. Begemann, M., Kashimawo, S. A., Heitjan, D. F., Schiff, P. B., Bruce, J. N., and Weinstein, I. B. (1998) *Anticancer Res. 18*, 2275−2282.
48. Jarvis, W. D., Turner, A. J., Povirk, L. F., Taylor, R. S., and Grant, S. (1994) *Cancer Res. 54*, 1707−1714.
49. Barnard, D. L., Huffman, J. H., Morris, J. L., Wood, S. G., Hughes, B. G., and Sidwell, R. W. (1992) *Antiviral Res. 17*, 63−77.
50. Le Panse, R., Coulomb, B., Mitev, V., Bouchard, B., Lebreton, C., and Dubertret, L. (1994) *Mol. Pharmacol. 46*, 445−451.
51. Nakanishi, N., Onozawa, S., Matsumoto, R., Kurihara, K., Ueha, T., Hasegawa, H., and Minami, N. (1995) *FEBS Lett. 368*, 411−414.
52. Meruelo, D., Lavie, G., and Lavie, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5230−5234.
53. Yanagihara, N., Tachikawa, E., Izumi, F., Yasugawa, S., Yamamoto, H., and Myamoto, E. (1991) *J. Neurochem. 56*, 294−298.
54. de Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A., and Bos, J. L. (1998) *Nature 396*, 474−477.
55. Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D., and Schachtele, C. (1993) *J. Biol. Chem. 268*, 9194−9197.
56. Meyer, T., Regenass, U., Fabbro, D., Alteri, E., Rösel, J., Müller, M., Carvatti, G., and Matter, A. (1989) *Int. J. Cancer 43*, 851−856.
57. Utz, I., Hofer, S., Regenass, U., Hilbe, W., Thaler, J., Grunicke, H., and Hofmann, J. (1994) *Int. J. Cancer 57*, 104−110.
58. Nishio, H., Ikegami, Y., Segawa, T., and Nakata, Y. (1994) *Gen. Pharmacol. 25*, 413−416.
59. Gopalakrishna, R., Chen, Z. H., and Gundimeda, U. (1992) *FEBS Lett. 314*, 149−154.
60. Zhou, T., Radaev, S., Rosen, B. P., and Gatti, D. L. (2000) *EMBO J. 19*, 4838−4845.
61. Ayesh, S., Shao, Y.-M., and Stein, W. D. (1996) *Biochim. Biophys. Acta 1316*, 8−18.
62. Boer, R., Diechtl, C., Ulrich, W. R., Maraceck, J. F., Prestwitch, G. D., Glossmann, H., and Striessnig, J. (1996) *Biochemistry 35*, 1387−1396.
63. Orlowski, S., Mir, L. M., Belehradek, J., Jr., and Garrigos, M. (1996) *Biochem. J. 317*, 515−522.

BI002453M