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A Novel Non-Natural Nucleoside That Influences P-Glycoprotein Activity and Mediates Drug Resistance[†]

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ABSTRACT: Multidrug resistance during cancer chemotherapy is commonly acquired by overexpression of the ATP binding cassette transporter, P-glycoprotein (P-gp). As such, inhibitors that target P-gp activity represent potential therapeutic agents against this form of drug resistance. This study evaluated the ability of various non-natural nucleosides that mimic the core structure of adenosine to modulate drug resistance by inhibiting the ATPase activity to P-gp. Of several analogues tested, only one novel non-natural nucleoside, 5-cyclohexylindolyl-2′-deoxyribose (5-CHInd), behaves as a P-gp inhibitor. Although 5-CHInd is an adenosine analogue that should block the binding of ATP, the non-natural nucleoside surprisingly stimulates the ATPase activity of P-gp in vitro. However, 5-CHInd is not an exportable substrate for P-gp as it is not transported across an MDCK-MDR1 monolayer. In addition, 5-CHInd differentially modulates MDR by decreasing or increasing the cytotoxicity of several chemotherapeutic agents. Although 5-CHInd displays variable activity in modulating the efflux of various drugs by P-gp, there is a correlation between changes observed in the drug-stimulated ATPase catalytic efficiency induced by 5-CHInd and its effect on drug efflux. The paradoxical behavior of 5-CHInd is rationalized within the context of contemporary models of P-gp function. In addition, the data are used to develop a predictive in vitro model for rapidly identifying potential drug−drug interactions with P-gp.

P-Glycoprotein (P-gp)[1] is a member of the ATP binding cassette (ABC) transporter family that is encoded by the *MDR1* gene (*1*, *2*). P-gp utilizes the energy derived from ATP binding and hydrolysis to export xenobiotics from the intracellular space. The normal physiological role of P-gp is to defend the cell against potentially toxic chemical entities. However, amplification of the *MDR1* gene leads to development of the multidrug resistance (MDR) phenotype in many types of cancers (*3*). This occurs as P-gp efficiently exports a number of structurally diverse chemotherapeutic agents, including vinca alkyloids, anthracyclines, taxanes, epipodophyllotoxins, and certain antibiotics (*4*). Indeed, amplification of *MDR1* strongly correlates with a poor prognosis in many forms of cancer (*5*). As a consequence, P-gp is a major clinical target in cancer chemotherapy (*6*).

At the cellular level, functional P-gp exists as a dimer containing two transmembrane domains and two nucleotide-binding domains (*7*). Substrate transport is an ATP-dependent process that follows a sequential mechanism. There are two major models that describe the role of ATP in substrate transport. The first model is the ATP switch model (*8*) which proposes that ATP binding drives dimerization of the nucleotide binding domains. This is followed by a change in the transmembrane domain conformation from an "open" conformation exposed to the cytoplasm to a "closed" conformation that is extracellular. Drug substrates are exported during the transition from the open to closed form. Hydrolysis of bound ATP resets the transmembrane domains back to the open conformation. The second model is termed the "occluded nucleotide" model (*9*). In this mechanism, ATP also drives dimerization of the nucleotide binding domains but does not result in a transition of the transmembrane domains from the open to closed form. Instead, there are two distinct ATP-bound states defined as a high-affinity occluded state and low affinity "non-occluded" state. At the start of the catalytic cycle, two ATP molecules are bound to the dimerized nucleotide binding domains in the non-occluded state with the transmembrane domains locked in an open conformation. The switch of one bound ATP to an occluded state promotes the change from the open to closed transmembrane domain conformation which coincides with drug substrate transport. The switch from non-occluded to occluded commits the nucleotide bound at that site to hydrolysis and subsequent dissociation. At that point, the other non-occluded nucleotide enters the catalytic cycle to stimulate another round of transport while the empty nucleotide binding site is reloaded with another nucleotide.

Despite the intricacies of the ATPase cycle, the most notable feature of P-gp is its enigmatic polyspecificity during drug transport. In this regard, P-gp facilitates the export of structurally diverse chemical entities such as vinblastine, doxorubicin, and digoxin. While these compounds do not share a common structural feature, they are all considered to be relatively hydrophobic. Indeed, this common feature is often used to justify why

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[1]Abbreviations: 5-CEInd, 5-cyclohexylindolyl-2′-deoxyribose; 5-CHInd, 5-cyclohexylindolyl-2′-deoxyribose; 5-CHITP, 5-cyclohexylindolyl-2′-deoxyribose triphosphate; 5-PhInd, 5-phenylindolyl-2′-deoxyribose; AP → BL, apical to basolateral; BL → AP, basolateral to apical; CsA, cyclosporine A; MDR, multidrug resistance; MDCK, Madin Darby Canine Kidney; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NIMH, National Institutes of Mental Health; PDSP, Psychoactive Drug Screening Program; P_{eff}, permeability coefficient; P-gp, P-glycoprotein; SAR, structure−activity relationship.

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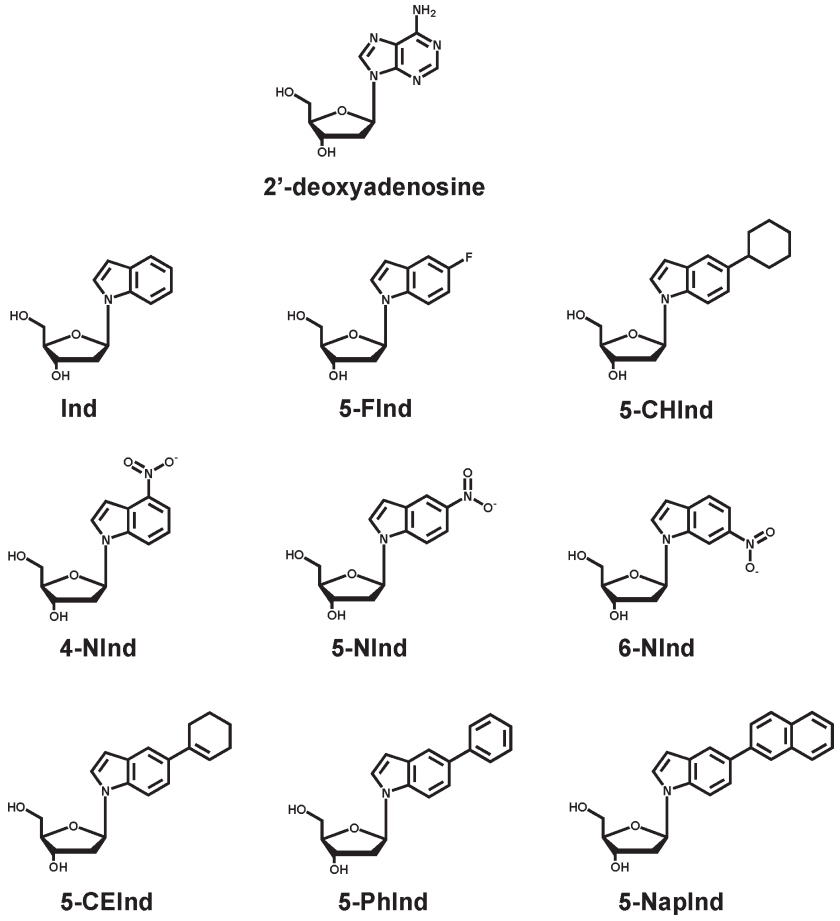

FIGURE 1: Structures of non-natural deoxyribose nucleosides used in this study.

many druglike compounds are substrates for P-gp. The most widely accepted model accounting for the polyspecificity of P-gp is the "hydrophobic vacuum cleaner" model originally proposed by Higgins and Gottesman (*10*). In this model, the hydrophobic nature of a potential P-gp substrate facilitates its partitioning into the lipid membrane. At this point, the substrate enters the transmembrane domains of P-gp and is transported out of the cell. Indeed, the recently described X-ray structure of P-gp is consistent with this model as there are four α-helices in the transmembrane domains of P-gp that could function as an entry portal for a substrate since they are directly accessible to the plasma membrane (*11*).

Attempts to generate inhibitors of P-gp have primarily focused on developing compounds that prevent entry of a substrate into the transmembrane domains of P-gp. This approach has met with some success as compounds such as VX-710 and S9788 can block the export of chemotherapeutic agents, including vinblastine and doxorubicin (*12*, *13*). However, there are significant limitations in this approach as most agents that inhibit drug export often simply serve as alternative substrates for P-gp. This can cause serious alterations in the pharmacokinetics during the coadministration of various drugs (*14*). As a result of this promiscuous activity, the development of an effective P-gp inhibitor is an important yet daunting challenge in drug design.

Since substrate transport depends upon ATP binding and hydrolysis, an alternative approach is to develop small molecule inhibitors that target the ATP binding site of P-gp. By blocking the binding of ATP, we can inhibit the drug transport capabilities of P-gp. While the rationale for this approach is sound, targeting the ATP binding site provides a new set of challenges that involve potency and selectivity. Indeed, there are numerous ATP binding proteins that could also be influenced by the binding of an ATP-like molecule. However, there are several examples in which a small molecule has been designed to selectively inhibit various ATP-dependent enzymes. One relevant example is Imatinib (Gleevec) which is used to treat chronic myelogenous leukemia by selectively targeting the kinase domain of the BCR−ABL fusion protein (*15*). In the case of targeting P-gp, the inhibitor tariquidar (*16*) displays high potency because of its ability to potentially function as a dual inhibitor by blocking the binding of drug substrate and ATP (*17*).

This report outlines our efforts to achieve similar pharmacological effects against P-gp by using various non-natural nucleosides (Figure 1) that mimic the core structure of adenosine. The goal is to use these nucleosides as pro-drugs that, after entry into the cell, would be converted into the corresponding triphosphates to inhibit the ATPase activity of P-gp. The validity of this approach was recently demonstrated by using a non-natural deoxynucleoside and corresponding triphosphate to inhibit the in vivo and in vitro activity of ATP-dependent replicative accessory proteins (*18*). In this report, a small library of structurally related nucleoside analogues was screened as inhibitors of P-gp activity using a cell-based assay. Of nine structurally related compounds tested, only 5-cyclohexylindolyl-2′-deoxyribose (5-CHInd) produced any ex vivo inhibitory effects against the transport activity of P-gp. Although the non-natural nucleoside influences the export of several known substrates of P-gp, the inhibitory effect is not caused by perturbing the ATPase activity of P-gp. In fact, 5-CHInd potently stimulates P-gp ATPase activity, displaying defined Michaelis−Menten kinetic behavior. The non-natural

nucleoside does not behave as a typical P-gp substrate as it is not transported across a cellular membrane under ex vivo conditions. In addition, 5-CHInd differentially influences the catalytic efficiency of drug-stimulated ATPase activity for several important chemotherapeutic agents. In this regard, an accurate correlation is observed between the effect of 5-CHInd of the ATPase activity of P-gp and its ability to affect the efflux capability of the drug transporter. Collectively, these data are used to develop a model directly correlating changes in the catalytic efficiency of drug-stimulated ATP hydrolysis activity with the modulation of drug resistance in MDR cells that can be used to predict multiple drug interactions with P-gp.

MATERIALS AND METHODS

Materials. All non-natural nucleosides and nucleotides were synthesized as previously described (*19*−*22*). Doxorubicin, paclitaxel, and cyclosporine A were purchased from Tocris Bioscience (Ellisville, MO). Verapamil, vinblastine, and colchicine were purchased from Sigma-Aldrich (St. Louis, MO). Calcein-AM was purchased from Calbiochem (San Diego, CA). All other chemicals were purchased from high-quality vendors. PEI-cellulose TLC plates were purchased from EM Science (Gibbstown, NJ). P-gp membranes were purchased from BD Biosciences (San Jose, CA). Transwell inserts (polycarbonate, 12-well, 11 mm diameter, 0.4 μm pores) were purchased from Corning Costar (Cambridge, MA). KB-V1, KB-3-1, MDCK-MDR1, and MDCK cells were a generous gift from M. Gottesman (National Cancer Institute, National Institutes of Health, Bethesda, MD). All cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA).

ATPase Assays and Measurement of Kinetic Parameters. ATPase activity was monitored by hydrolysis of [γ-^{32}P]ATP. Reaction buffer consisted of 1 mM ATP, 50 mM Tris-MES, 2 mM EGTA, 2 mM DTT, 50 mM KCl, and 5 mM sodium azide. All reactions were conducted at 37 °C in triplicate. Reactions were initiated by addition of 5 μg of P-gp membranes and quenched at 5 and 20 min in an equal volume of 10% SDS. Quenched samples were analyzed by thin layer chromatography on PEI-cellulose plates using 0.6 M KH_2PO_4 (pH 3.5). Imaging of thin layer chromatography was accomplished using a Packard Cyclone PhosphorImager. The ratio of free $^{32}P_i$ to nonhydrolyzed [γ-^{32}P]ATP was multiplied by the final concentration of ATP to yield the total product concentration. Product formation in the absence of enzyme was assessred and subtracted from all measurements. Initial velocities were obtained by fitting the data to eq 1.

$$y = mx + b \quad (1)$$

where y is the product concentration, x is time, m is the slope, and b is the y-intercept. K_m and V_{max} values were obtained by fitting initial velocities to eq 2.

$$v = (V_{max}[S])/(K_m + [S]) \quad (2)$$

where v is the velocity, V_{max} is the maximal velocity, [S] is the substrate concentration, and K_m is the Michaelis−Menten constant.

Cell Culture Techniques. All cell lines were generously provided by M. Gottesman. All cells were cultured at 37 °C in humidified air with 5% CO_2. KB-3-1 cells were grown and maintained in Dulbecco's modified Eagle's medium with 10% FBS, 2 mM L-glutamine, 4500 mg/L glucose, 110 mg/L sodium pyruvate, and 100 μg/mL penicillin/streptomycin. The doubling time was approximately 24 h. Cells were split weekly at a ratio of 1:4. KB-V1 cells were grown and maintained under the same conditions with the addition of 100 nM vinblastine. MDCK cells were grown and maintained in Minimal Essential Medium with 10% FBS, 2 mM L-glutamine, and 100 μg/mL penicillin/streptomycin. Cells were split twice weekly at a ratio of 1:10. MDCK-MDR cells were grown and maintained under the same conditions with the addition of 200 nM colchicine.

Monolayer Efflux Experiments. MDCK-MDR and MDCK cells were seeded in polycarbonate transwell membranes at a density of 300000 cells/cm^2. Medium was changed every other day, and monolayers were ready for experimentation 7 days after plating. For a typical transport experiment, monolayers were washed and preincubated with transport buffer (Hank's balanced salt solution) for 30 min. Transport was initiated by the addition of drug to the donor well, and assays were conducted for 60 min. Drug transport was assessed in the BL → AP direction, and final drug concentrations in the donor and receiver wells were quantified via spectroscopic measurements using the following parameters (for 5-CHInd, λ_{max} = 266 nm and ε_{266} = 6840 $M^{-1}cm^{-1}$; for calcein-AM, λ_{max} = 496 nm and ε_{496} = 6000 M^{-1} cm^{-1}; for rhodamine 123, λ_{max} = 500 nm and ε_{500} = 75000 M^{-1} cm^{-1}). Permeability coefficients were obtained from eq 3:

$$P_{eff} = 1/AC_o(dQ/dt) \quad (3)$$

where P_{eff} is the permeability coefficient, A is the membrane surface area, C_o is the initial drug concentration in the donor well, and dQ/dt is the amount of drug transported over a given period of time.

Measure of in Vivo Cytotoxicity. The cytotoxicity of 5-CHInd and other chemotherapeutic agents was quantified using a standard MTT assay. Cells were seeded at a density of 20000 cells/well in a 96-well plate. Cells were incubated for 24 h after plating to ensure proper adhesion. At the time of drug treatment, 10% FBS medium was replaced with 2.5% FBS medium, and the DMSO content did not exceed 0.4%. Raw data were normalized to 100% viability (no treatment), and LD_{50} values were obtained through a fit of the data to eq 4.

$$y = 100/[1 + (LD_{50}/I)^n] \quad (4)$$

where y is the fractional viability, I is the concentration of drug, LD_{50} is the concentration of drug that causes 50% cell death compared to cells treated with vehicle, and n is the Hill coefficient.

RESULTS

Identification of 5-CHInd as a Modulator of P-gp Activity. Conventional P-gp inhibitors are generally hydrophobic compounds that target the drug export site of the transporter. Our attempts to develop an alternative class of inhibitor focused on targeting the ATP binding site of P-gp. We used the non-natural deoxyribose nucleosides depicted in Figure 1 since they mimic the core structure of adenosine. In addition, we previously demonstrated that the triphosphate form of certain non-natural nucleosides can act as either surrogates of dATP (*23*) or inhibitors of ATP-dependent enzymes (*18*). To test the ability of these non-natural nucleosides to inhibit P-gp, we submitted them to the NIMH PDSP screening facility (http://pdsp.med.unc.edu/indexR.html). This facility screens compounds using a cell-based assay that measures the accumulation of calcein-AM, a highly

selective substrate for P-gp, in Caco-2 cells. In cells that overexpress P-gp, the nonfluorescent calcein-AM is actively exported and thus generates a weak fluorescence signal. However, inhibition of P-gp causes calcein-AM to accumulate within the cell. Hydrolysis by intracellular esterases converts calcein-AM into calcein which is highly fluorescent, and the production of a fluorescence signal indicates a functional inhibition of drug efflux. From a screen of these structurally related non-natural nucleosides, 5-CHInd was identified as the only compound that inhibited the efflux of calcein-AM with an efficacy equal to that of cyclosporine A (CsA), a potent P-gp inhibitor (data not shown).

Effects of 5-CHInd on P-gp ATPase Activity. Although 5-CHInd inhibited calcein-AM efflux, this effect could be caused by several mutually exclusive mechanisms. These mechanisms include the ability of the non-natural nucleoside to act as a competitive inhibitor for ATP binding, as an alternative substrate for efflux, or as a nontransported substrate. To differentiate among these possibilities, we measured the effect of 5-CHInd on the ATPase activity of P-gp. If 5-CHInd acts as an ATP competitive inhibitor, then the low basal ATPase activity of P-gp should be reduced even further by the addition of increasing concentrations of 5-CHInd. Conversely, the ATPase activity of P-gp should be stimulated if 5-CHInd behaves as either an alternative or nontransported substrate.

The ATPase activity of P-gp was measured in the presence of increasing concentrations of 5-CHInd using a single, fixed concentration of 1 mM ATP. Surprisingly, the rate of ATP hydrolysis increased as a function of increasing concentrations of 5-CHInd. Initial velocities were plotted as a function of 5-CHInd concentration (Figure 2A), and the data were fit to the Michaelis−Menten equation to define K_m and V_{max} values. The data illustrated in Figure 2A show that 5-CHInd potently stimulates P-gp ATPase activity with a K_m of 60 ± 10 nM. The selectivity for 5-CHInd was interrogated by testing the ability of other structurally related analogues such as 5-CEInd and 5-PhInd to influence the ATPase activity of P-gp. As illustrated in Figure 2B, neither compound displays any stimulatory or inhibitory activity against the ATPase activity of P-gp. Other non-natural nucleosides also do not stimulate the ATPase activity of P-gp (data not shown). These results are consistent with data obtained using the calcein-AM screening assay (vide supra), indicating that these analogues do not interact with P-gp.

To compare the potency of 5-CHInd with those of other classical P-gp substrates, K_m and V_{max} values were measured for compounds such as paclitaxel, vinblastine, doxorubicin, colchicine, verapamil, and cyclosporine A (Table 1). While all of these agents stimulate P-gp ATPase activity, none is as potent as 5-CHInd. In fact, the potency for these substrates varies by several orders of magnitude (Table 1), with 5-CHInd being the most potent substrate (K_m = 60 nM) and colchicine being the least potent (K_m = 37 μM). Despite vast differences in these K_m values, the V_{max} for all substrates exhibited less variation with an average value of ~5 nmol of P_i min^{-1} (mg of protein)$^{-1}$. Collectively, these data strongly suggest that 5-CHInd does not behave as a competitive inhibitor against ATP but rather as an alternative or a nontransported substrate.

5-CHInd Is a Nontransported Substrate of P-gp. To differentiate between these mechanisms, the ability of P-gp to transport 5-CHInd was directly measured using the monolayer efflux assay with the MDCK and MDCK-MDR1 cell lines. Substrate transport was assessed via calculation of the P_{eff} in the basolateral (BL) to apical (AP) direction and vice versa. In general, the ratio of P_{eff} values in the BL → AP to AP → BL directions define the degree of P-gp-mediated transport (*24*). Since P-gp is localized to the surface of these cells, positive ratios indicate polarized P-gp-mediated transport. To define the degree

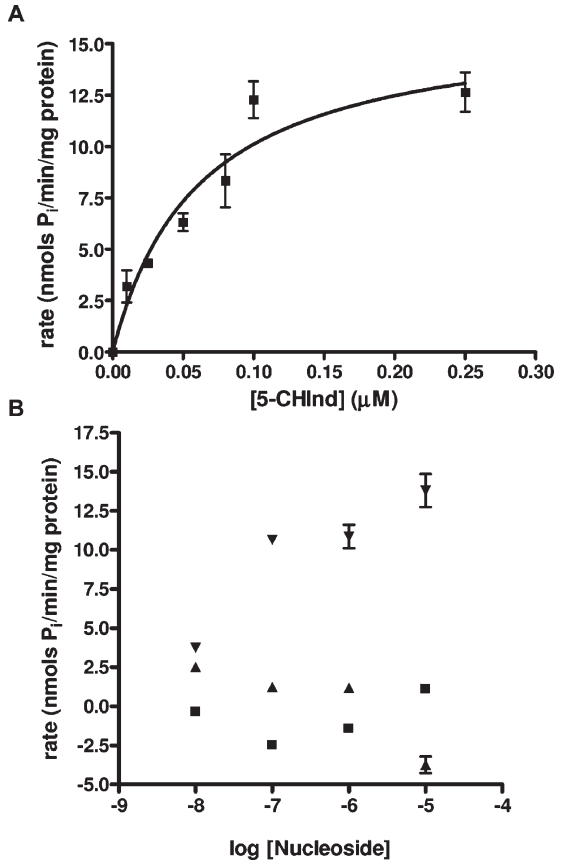

FIGURE 2: Stimulation of P-gp ATPase activity by non-natural nucleosides. (A) Inside-out P-gp-enriched membranes were used for ATPase measurements, and the Michaelis−Menten kinetic parameters, K_m and V_{max}, were obtained by stimulating P-gp ATPase activity with increasing concentrations of 5-CHInd. The ATP concentration was fixed at 1 mM, and initial velocities were measured over 20 min. K_m = 60 ± 18 nM, and V_{max} = 16 ± 0.2 nmol of Pi min^{-1} (mg of protein)$^{-1}$. (b) Stimulation of P-gp ATPase activity in inverted P-gp-enriched membranes by increasing concentrations of 5-CHInd (▼), 5-CEInd (▲), and 5-PhInd (■).

Table 1: Kinetic Parameters for the Stimulation of ATPase Activity by Drug Substrates

drug substrate	K_m (μM)[a]	V_{max} [nmol of P_i min^{-1} (mg of protein)$^{-1}$][a]	V_{max}/K_m [nmol of P_i min^{-1} (mg of protein)$^{-1}$ μM^{-1}]
5-CHInd	0.06 ± 0.01	16 ± 0.2	266.7 ± 39.0
calcein-AM	3.1 ± 0.5	46 ± 2	14.8 ± 1.5
vinblastine	0.20 ± 0.03	17.5 ± 0.5	87.5 ± 2.0
colchicine	37 ± 8	4.0 ± 0.2	0.1 ± 0.015
doxorubicin	0.85 ± 0.17	3.5 ± 0.2	4.1 ± 0.5
paclitaxel	0.9 ± 0.2	9.5 ± 1.0	10.6 ± 1.5
verapamil	4.0 ± 1.7	16.5 ± 2.5	4.1 ± 1.0
cyclosporine A	0.40 ± 0.16	7.0 ± 1.0	17.5 ± 1.5

[a] K_m and V_{max} values are means ± the standard error of the mean (SEM) calculated from at least three independent experiments. Initial velocities were measured in the presence of a fixed ATP concentration (1 mM) and varying drug concentrations. K_m and V_{max} values were obtained by fitting the initial velocities to the Michaelis−Menten equation.

Table 2: Permeability Coefficients of Various P-gp Substrates across MDCK and MDCK-MDR Monolayers

MDCK-MDR1	BL → AP:AP → BL[a]
10 μM 5-CHInd[b]	11.1 ± 2.5
10 μM 5-CHInd with 10 μM adenosine[c]	1.6 ± 0.2
3 μM rhodamine 123[d]	72.7 ± 15.3
4 μM calcein-AM[e]	2940 ± 120
4 μM calcein-AM with 10 μM 5-CHInd	0.98 ± 0.18
MDCK	**BL → AP:AP → BL**
10 μM 5-CHInd	8.7 ± 1.3
10 μM 5-CHInd with 10 μM adenosine[c]	2.3 ± 0.6
3 μM rhodamine 123	1.2 ± 0.2
4 μM calcein-AM	2.6 ± 0.3

[a]Values are mean ± SEM calculated from three independent experiments. [b]5-CHInd spectroscopic detection parameters: λ = 260 nm, and ε = 6843 M^{-1} cm^{-1}. [c]5-CHInd and adenosine were measured at λ = 260 nm and ε = 6843 M^{-1} cm^{-1} (5-CHInd) or ε = 15200 M^{-1} cm^{-1} (adenosine). [d]Calcein-AM spectroscopic detection parameters: λ = 496 nm, and ε = 6000 M^{-1} cm^{-1}. [e]Rhodamine 123 spectroscopic detection parameters: λ = 500 nm, and ε = 75000 M^{-1} cm^{-1}.

of transport, these ratios are compared against values obtained with known substrates such as calcein-AM and rhodamine 123. Using the MDCK-MDR1 cell line, calcein-AM and rhodamine 123 display P_{eff} ratios of 2940 and 72.7, respectively. These high values validate the fact that they are indeed substrates of P-gp. In contrast, the P_{eff} ratio for 5-CHInd in the MDCK-MDR1 cell line is significantly lower [11.1 (Table 2)] and suggests that the non-natural nucleoside is not exported by P-gp. In addition, a nearly identical P_{eff} ratio is obtained using the MDCK cell line that does not overexpress *MDR1* (Table 2). To further validate the idea that 5-CHInd is a nontransported substrate, we tested the ability of 5-CHInd to block the transport of calcein-AM across MDCK-MDR1 monolayers. As predicted, 5-CHInd completely blocks the transport of calcein-AM as its P_{eff} ratio is reduced from ~3000 in the absence of 5-CHInd to ~1 in the presence of 10 μM non-natural nucleoside (Table 2).

5-CHInd Is Imported by the Nucleoside Transporter. The low P_{eff} ratio of 5-CHInd could reflect passive diffusion through the monolayer. Alternatively, 5-CHInd could be imported into the cell by the action of a nucleoside transporter. Measuring the effects of adenosine on the permeability of 5-CHInd assessed this possibility since adenosine is a high-affinity substrate for the nucleoside transporter (*25*) that should block the transport of other potential substrates such as 5-CHInd. The data provided in Table 2 validate this mechanism as the P_{eff} ratio for 5-CHInd decreases from 11.1 in the absence of adenosine to 1.56 in its presence. A similar result is observed using the MDCK cell line that does not overexpress P-gp (Table 2). Collectively, these results indicate that 5-CHInd is imported via a nucleoside transporter rather than via passive diffusion as expected for a typical hydrophobic compound.

Correlating ATPase Activity with the Modulation of Drug Export. The data suggest that 5-CHInd is a nontransported substrate that stimulates the ATPase activity of P-gp. If correct, then the binding of 5-CHInd should block the binding of a substrate such as calcein-AM. The resulting inhibition would be reflected in a decrease in the V_{max}/K_m value in the presence of 5-CHInd. For a competitive inhibitor (*26*, *27*), the decrease in the overall catalytic efficiency would be caused by an increase in the K_m for calcein-AM while the V_{max} for the reaction remains

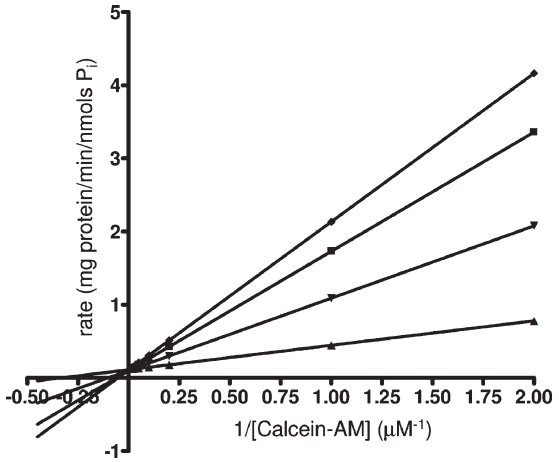

FIGURE 3: 5-CHInd is a competitive inhibitor of P-gp. Double-reciprocal plot of rate vs calcein-AM concentration at several fixed concentrations of 5-CHInd. 5-CHInd concentrations were set as follows: no inhibitor (▲), 10 nM 5-CHInd (▼), 20 nM 5-CHInd (■), and 30 nM 5-CHInd (◆). The K_i value of 6.5 ± 0.7 μM was determined through a fit of the data to the equation rate = $(V_{max}[S])/K_m(1 + [I]/K_i) + [S]$.

unchanged. To validate this hypothesis, initial velocity data for ATP hydrolysis were obtained via variation of the concentration of calcein-AM at several different concentrations of 5-CHInd. The double-reciprocal plot provided in Figure 3 clearly indicates that 5-CHInd behaves as a competitive inhibitor against calcein-AM. The K_i value of 6.5 ± 0.7 nM measured for 5-CHInd is ~9-fold lower than the measured K_m value of 60 nM. The difference between K_i and K_m values suggests a two-step binding mechanism for 5-CHInd in which there is a potential conformational change step that exists between the binding of 5-CHInd to P-gp and ATP hydrolysis. Further experimentation is needed to validate this claim. Regardless, these data are consistent with 5-CHInd behaving as a competitive inhibitor against calcein-AM and thus validate the results obtained using the PDSP screening facilities (vide supra).

V_{max}/K_m values for other P-gp substrates were measured in the absence and presence of 5-CHInd. These values, summarized in Table 3, are useful in defining the potential interactions of 5-CHInd with the second substrate. This analysis is based on comparing the ratio of the V_{max}/K_m value measured with drug and 5-CHInd $\{V_{max}/K_{m(drug+5\text{-}CHInd)}$ compared to the V_{max}/K_m for drug alone $[V_{max}/K_{m(drug)}]\}$. In this analysis, a ratio of <1 represents a decrease in activity caused by 5-CHInd inhibition while a ratio of >1 reflects an increase in activity that could represent cooperative efflux. As expected, the ratio using 5-CHInd against calcein-AM as the substrate is <1. Nearly identical results are obtained using CsA as the inhibitor to influence the ATPase activity by calcein-AM. In fact, identical ratios of ~0.14 indicate that both 5-CHInd and CsA inhibit the efflux of calcein-AM.

We next investigated the effect of 5-CHInd on the efflux of vinblastine, doxorubicin, colchicine, paclitaxel, and verapamil by defining these ratios in the catalytic efficiencies in ATPase activity. As reported in Table 3, the ratio using paclitaxel is significantly less than 1, indicating that 5-CHInd also inhibits its efflux. However, 5-CHInd does not behave as a universal inhibitor since the ratios for vinblastine and doxorubicin are greater than 1. These data suggest that the non-natural nucleoside enhances their export by binding to at least two mutually exclusive sites that functionally interact with each other.

Table 3: Effects of 5-CHInd on the Catalytic Efficiency of Drug-Stimulated P-gp ATPase Activity

	$V_{max}/K_{m(drug)}$ [nmol of P_i min^{-1} (mg of protein)$^{-1}$ μM^{-1}]	$V_{max}/K_{m(drug+5\text{-}CHInd)}$[a][nmol of P_i min^{-1} (mg of protein)$^{-1}$ μM^{-1}]	$[V_{max}/K_{m(drug+5\text{-}CHInd)}]/[V_{max}/K_{m(drug)}]$
calcein-AM	2.93	0.42	0.143
calcein-AM with CsA[b]	2.93	0.38	0.131
vinblastine	16.7	64.7	3.9
colchicine	0.021	0.023	1.09
doxorubicin	0.86	5.5	6.4
paclitaxel	2.1	> 0.02	> 0.01
verapamil	0.80	0.84	1.05

[a]The 5-CHInd concentration for all experiments was 60 nM. [b]Cyclosporine A (CsA) at 400 nM was used in place of 5-CHInd.

Finally, the inclusion of 5-CHInd has no effect on the V_{max}/K_m values for colchicine or verapamil. These data suggest a lack of interaction between the binding of 5-CHInd and colchicine or verapamil.

5-CHInd Modulates Drug Resistance in MDR-Positive Cells. The differential effects of 5-CHInd on the ATPase activity of P-gp could be used to influence resistance to the effects of certain drugs in cell lines that overexpress P-gp. To test this hypothesis, we used an MDR-positive cell line (KB-V1 *MDR1* +/+) to evaluate the ability of 5-CHInd to alter the cytotoxic effects of various anticancer agents. Cytotoxicity was assessed using an MTT assay that measures the loss of cell viability as a function of drug concentration. Since 5-CHInd is cytotoxic at concentrations of > 50 μM (vide infra),[2] changes in drug resistance were evaluated by comparing the LD_{50} value for a chemotherapeutic agent in the absence and presence of a sublethal dose of 5-CHInd. As shown in Figure 4A, the LD_{50} for paclitaxel decreases in the presence of 5-CHInd, thereby sensitizing the MDR-positive cells to the cytotoxic effects of the anticancer agent. The ability of 5-CHInd to potentiate the effects of paclitaxel correlates with the inhibition of ATPase activity. An opposite effect is observed when vinblastine or doxorubicin is combined with 5-CHInd (Figure 4A) as the cells become more resistant to their cytotoxic effects in the presence of 5-CHInd. These data suggest that 5-CHInd enhances their efflux to further enhance resistance. In this case, the ability of 5-CHInd to protect the cell against the effects of these chemotherapeutic agents correlates with ratios greater than 1 measured in the catalytic efficiencies in ATPase activity. Finally, the presence of 5-CHInd did not influence the LD_{50} values for colchicine. The lack of a cellular effect is expected since no change in the catalytic efficiency ratio for ATP hydrolysis was detected in the presence of 5-CHInd. Collectively, there is an excellent correlation between the ability of 5-CHInd to modulate drug resistance in cell lines overexpressing P-gp and the in vitro-derived catalytic activity ratios for the ATPase activity of P-gp with these agents.

To further characterize the MDR reversing effects of 5-CHInd, KB-V1 cells were treated with increasing concentrations of paclitaxel and a fixed concentration of 5-CHInd (50 μM) for 48 h. Under these conditions, the cytotoxicity of paclitaxel is increased ~10-fold by a sublethal dose of 5-CHInd (Figure 4B). This potentiating effect is not observed using the isogenic cell line, KB-3-1, that does not overexpress P-gp (Supporting Information 1). This result confirms that potentiation by 5-CHInd is caused by functional inhibition of P-gp. Furthermore,

[2]Treatment with 10 μM 5-CHInd was performed over a 72 h period. Treatment with 50 μM 5-CHInd was performed over a 48 h period. These concentrations were the maximal nontoxic doses for each given time period.

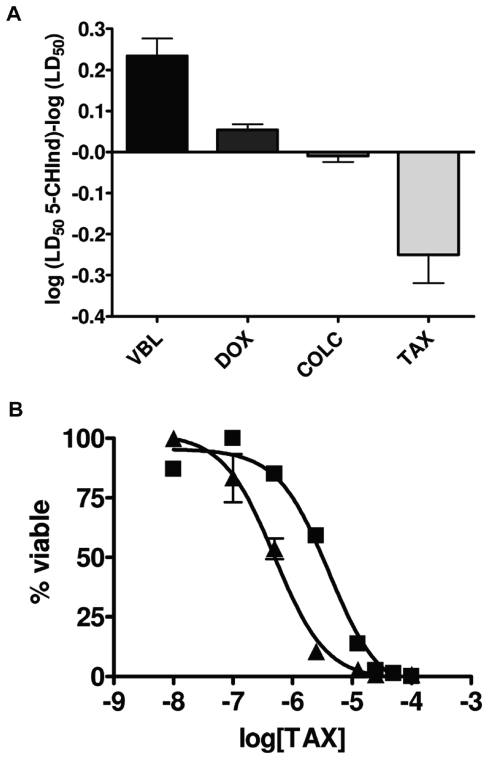

FIGURE 4: Modulation of drug resistance by 5-CHInd in KB-V1 cells. (A) Effects of 10 μM 5-CHInd over 72 h on the log (LD_{50}) values of vinblastine (VBL), doxorubicin (DOX), colchicine (COLC), and paclitaxel (TAX). (B) Potentiation effects of 50 μM 5-CHInd over 48 h on the LD_{50} of paclitaxel (TAX): for no treatment (■), LD_{50} = 4.0 ± 0.24 μM; for 50 μM 5-CHInd (▲), LD_{50} = 0.49 ± 0.03 μM.

the related structural analogues, 5-CEInd and 5-PhInd, had no significant effects on MDR phenotype (Supporting Information 2) and again highlight the unique selectivity of 5-CHInd as a modulator of P-gp activity.

Cytotoxicity of 5-CHInd. As mentioned above, 5-CHInd displays cytotoxic effects in a dose- and time-dependent manner. The LD_{50} value of 5-CHInd was measured using the KB-3-1 and KB-V1 cell lines in which cells were treated with concentrations of 5-CHInd that range over several log units (from 0.001 to 200 μM). At 72 h post-treatment, cell viability was assessed using the MTT assay. The LD_{50} value for 5-CHInd in the MDR-positive cell line is 44 ± 5 μM. An identical LD_{50} value of 45 ± 3 μM was measured in the isogenic cell line not overexpressing P-gp (KB-3-1 *MDR* −/−) (Supporting Information 3). The identity in LD_{50} values indicates that the cytotoxic effect of 5-CHInd is independent of P-gp and again validates the idea that the non-natural nucleoside is not a substrate for the drug transporter.

DISCUSSION

A goal of molecular medicine is to develop small molecule inhibitors against therapeutically important targets. P-gp is one such important therapeutic target as it functions under certain conditions to generate an MDR phenotype by actively transporting various chemotherapeutic agents out of a cell. However, developing an efficient and selective inhibitor against P-gp proves to be a difficult challenge because of the complexities in the mechanism by which P-gp catalyzes the drug efflux. In particular, most attempts are directed toward targeting the site responsible for drug export. In this report, we investigate an alternative approach using non-natural nucleosides as potential inhibitors of P-gp that target its ATP-binding site. The key findings of this paper include the following: (1) the identification of a novel non-natural nucleoside that modulates P-gp activity by stimulating ATPase activity without being a transportable substrate, (2) the ability of the non-natural nucleoside to influence the export of other drugs either by competitive inhibition or through cooperative efflux, and (3) the development of a model correlating changes in the catalytic efficiency of drug-stimulated ATP hydrolysis activity with the modulation of drug resistance in MDR cells. Each point is discussed to highlight their importance.

The initial goal of this work was to identify inhibitors of P-gp by targeting its ATP binding site. In this regard, we used a series of substituted indolyl 2′-deoxynucleosides as a novel class of purine nucleoside analogues that structurally mimic the basic structure of adenosine and that function as mimetics of ATP and dATP (*19*−*22*). Initial cell-based screening identified 5-CHInd was the only inhibitor of P-gp export activity among this group of structurally related non-natural nucleosides. In spite of its inhibitory effects on drug efflux, 5-CHInd does not behave as a competitive inhibitor targeting the ATP binding site of P-gp as initially predicted. Instead, 5-CHInd stimulates the in vitro ATPase activity of P-gp with high potency as exhibited by a very low K_m value of 60 nM. Monolayer efflux experiments validate that 5-CHInd is not exported from cells overexpressing P-gp. This phenomenon is not limited to 5-CHInd since Polli and coworkers reported similar results using drugs such as verapamil, ketoconazole, and nifedapine (*24*). In addition, 5-CHInd displays identical LD_{50} values in both MDR-positive (KB-V1) and MDR-negative (KB-3-1) cell lines. Although 5-CHInd is not exported by P-gp, it does inhibit the efflux of known P-gp substrates such as calcien-AM and paclitaxel. These data collectively support a model in which 5-CHInd behaves as a nontransportable substrate for P-gp.

From a molecular perspective, it is surprising that closely related analogues of 5-CHInd such as 5-CEInd and 5-PhInd fail to elicit any effect on P-gp activity. This result is unexpected since all three compounds are extremely hydrophobic ($\log P_{5\text{-CHInd}} = 3.63$, $\log P_{5\text{-CEInd}} = 3.15$, and $\log P_{5\text{-PhInd}} = 3.31$) (*21*, *22*) and should, according to the well-established hydrophobic vacuum cleaner model, behave as substrates for P-gp. In addition, the recently published X-ray structure of P-gp describes the drug binding cavity as a large chamber possessing a potential volume of 6000 Å^3 (*11*). The large size of the cavity can easily accommodate the binding of all three compounds. A possible mechanism for explaining this dichotomy is that the larger volume of the cyclohexyl ring of 5-CHInd (96.1 Å^3) makes more favorable contacts with hydrophobic residues that reside within this large binding cavity.[3] The net effect of these favorable contacts is a slow dissociation rate constant that translates into high binding affinity. In contrast, the smaller volumes of the cyclohexene (91.2 Å^3) and phenyl (82.8 Å^3) groups and/or differences in their overall shape and configuration could provide less favorable interactions which would be reflected in poor affinity. Another possibility is with respect to differences in solvation energies between 5-CHInd (−4.23 kJ/mol) compared to 5-CEInd (−5.11 kJ/mol) and 5-PhInd (−6.59 kJ/mol). Although hydrophobicity and desolvation are generally used interchangeably, each term represents a different biophysical feature that can influence the binding and transport of certain drugs. Hydrophobicity defines the tendency of a molecule to repel water, whereas desolvation energy defines the quantity of energy required to remove water from a molecule. The lower solvation energy of 5-CHInd compared to those of 5-CEInd and 5-PhInd suggests that P-gp could more easily displace water molecules surrounding 5-CHInd to accommodate binding. In this mechanism, the greater π-electron surface area and induced dipole moments associated with 5-CEInd and 5-PhInd could form a more defined solvation center that presents a larger barrier for desolvation. This would provide a kinetic barrier that hinders their binding. Indeed, this feature may also explain why other analogues such as 5-NInd and 5-NapInd that also contain large π-electron surface areas do not interact with P-gp. Since 5-CHInd lacks significant π-electron density or dipole moment at the 5-position of the indolyl ring, it circumvents this desolvation step. This mechanism is being further explored via development of other non-natural nucleosides that contain differing degrees of π-electron density and solvation energies.

Regardless of these possibilities, it is clear that 5-CHInd binds to and modulates the activity of drug efflux. In fact, the ability of 5-CHInd to block the transport of compounds such as calcein-AM and paclitaxel suggests that all three compounds can occupy the same binding site. This provides another interesting conundrum as there are few, if any, structural similarities between 5-CHInd and either calcein-AM or paclitaxel (Supporting Information 4). This feature exemplifies the polyspecificity displayed by P-gp which has hindered the development of "rules" that accurately define the interactions of P-gp with a potential substrate. In fact, the classification of most P-gp substrates is based upon the ability of one compound to influence the export of another. For example, Ling and colleagues were among the first to report that rhodamine 123 and Hoechst 33342, two well-known P-gp substrates, display a synergistic increase in their rates of efflux when co-administered (*28*). On the basis of these observations, they assigned a potential binding site corresponding to each probe ("H" for Hoechst 33342 and "R" for rhodamine 123). Other substrates have since been classified as "H-site" or "R-site" on the basis of their ability to influence the efflux rate of either probe.

At face value, the paradoxical behavior of 5-CHInd toward various chemotherapeutic substrates is consistent with this model as the non-natural nucleoside behaves like an H-site drug by stimulating the ATP hydrolysis activity and transport capabilities of drugs such as vinblastine and doxorubicin. In addition, 5-CHInd and paclitaxel appear to compete for the same binding site since 5-CHInd squelches any increase in ATPase activity caused by paclitaxel. Finally, 5-CHInd inhibits the transport of the taxane to potentiate its cytotoxic effects.

Unfortunately, 5-CHInd does not always obey this model. In particular, 5-CHInd has no effect on the ex vivo cytotoxicity of

[3]Values corresponding to volumes and desolvation energies of these non-natural nucleobases were calculated using Spartan '04.

colchicine or its ability to stimulate ATP hydrolysis. These data contradict the R- and H-site model since colchicine and 5-CHInd are both predicted to be H-site drugs. This effect is not limited to 5-CHInd as other compounds are reported to display similar effects in modulating the efflux of other compounds (*29*, *30*). For example, Kondratov et al. observed similar effects toward modulating P-gp by QB102, a compound identified from chemical library screening (*30*). In this case, QB102 increases resistance to anthracyclines while it also decreases resistance to taxanes and vinca alkaloids. Sterz et al. recently observed a similar phenomenon with compounds that are structurally related to QB102 (*29*). Collectively, these data suggest that the R- and H-site model cannot be used to unambiguously and accurately predict the dynamics of interactions of multiple drugs with P-gp.

Although the molecular mechanism accounting for the paradoxical behavior of 5-CHInd remains undefined, the data provided in this report are used to develop a predictive model to evaluate the functional interactions of two druglike molecules with P-gp. This model is based on correlating the influence of 5-CHInd on the ATPase activity of P-gp with the effect of 5-CHInd on P-gp transport activity. Specifically, the ratio of V_{max}/K_m values in ATP hydrolysis for a substrate measured in the absence or presence of 5-CHInd provides an accurate indication of drug−drug interactions. For example, the ratio of less than 1 measured using paclitaxel indicates a decrease in ATPase activity, and this correlates with an inhibitory effect on its efflux as confirmed by the ability of 5-CHInd to potentiate its cytotoxic effects in a cancer cell overexpressing P-gp. Likewise, drugs such as vinblastine and doxorubicin display ratios greater than 1 with 5-CHInd which correlate with an enhancement in their export as demonstrated by the ability of 5-CHInd to increase the cellular resistance to vinblastine in cells overexpressing P-gp. The data summarized in Figure 5A show a clear correlation between the effects of 5-CHInd on drug-stimulated ATPase activity and the MDR phenotype in cell culture for drugs such as paclitaxel, colchicine, and vinblastine. As illustrated in Figure 5B, this correlation provides a valuable way to predict either positive or negative end points of MDR modulation by the ability of 5-CHInd to influence drug efflux of various compounds. Although this model is based on the ability of 5-CHInd to influence the efflux of various drugs, it can easily be extrapolated to monitor other drug−drug interactions with P-gp. For example, Lagas and co-workers reported an increase in the level of dasatinib accumulation in the brain after treatment with the P-gp inhibitor elacridar (*31*). In this scenario, one would predict that elacridar leads to a decrease in the dasatinib-stimulated ATPase activity of P-gp, leading to a V_{max}/K_m ratio of < 1 that is caused primarily by an increase in the K_m for dasatinib. Another example is the P-gp-mediated increase in ivermectin plasma concentration upon multiple treatments with ketoconazole (*32*). Ivermectin has been implicated in many P-gp−drug interactions (*33*) and thus represents an ideal candidate for defining these interactions as well as validating the predictive model described here. In general, the ability to predict if one therapeutic agent influences the export (or retention) of another drug has important clinical ramifications, especially within the context of diseases such as cancer in which multidrug regimens are routinely employed to combat the diseased state.

This type of analysis also has several practical advantages over conventional assays that are used to probe drug−drug interactions with P-gp. For example, the calcein-AM efflux assay is rapid and amenable to high-throughput screening. However, as

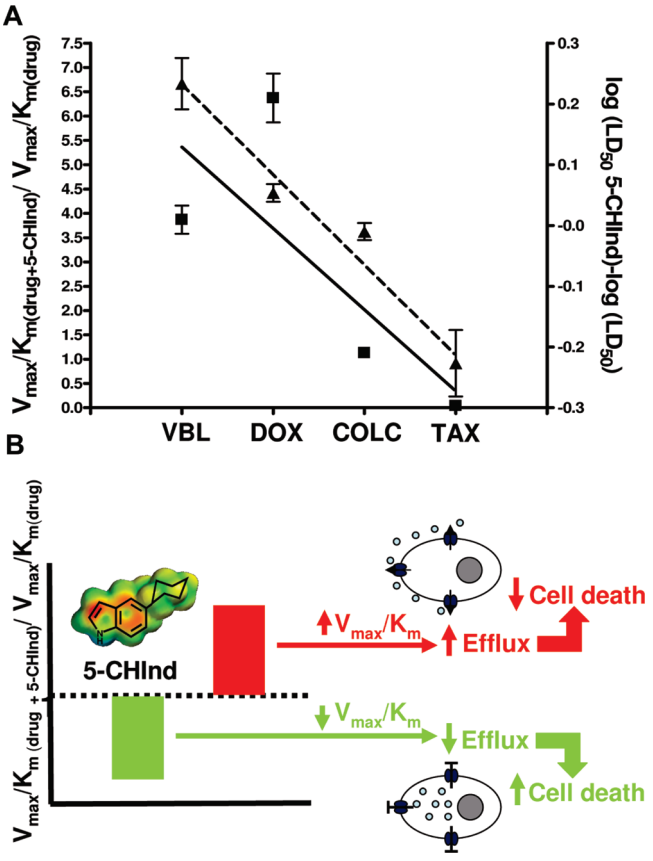

FIGURE 5: (A) Correlation of ATPase V_{max}/K_m ratios with modulation of MDR phenotype. The following drugs were used in this study: vinblastine (VBL), doxorubicin (DOX), colchicine (COLC), and paclitaxel (TAX). The left axis (■) indicates changes in drug-stimulated ATPase V_{max}/K_m ratios in the presence of 60 nM 5-CHInd. The right axis (▲) indicates changes in log(LD_{50}) for cytotoxic drugs in KB-V1 cells in the presence of 10 μM 5-CHInd over 72 h. (B) Model correlating the effect of a compound such as 5-CHInd on the ATPase activity of P-gp with drug resistance in MDR-positive cells. In this model, the influence on drug resistance reflects enhanced export or retention of the drug of interest. V_{max}/K_m ratios greater than 1 indicate an increase in P-gp activity which generates an increase in drug resistance. Conversely, V_{max}/K_m ratios of less than 1 reflect a decrease in P-gp activity that correlates with a decrease in drug resistance.

demonstrated here, it provides only qualitative information regarding drug efficacy and potency. In addition, it cannot distinguish between an inhibitor and an alternative substrate. The monolayer efflux assay provides a more quantitative approach to measuring substrate transport across a monolayer of cells. However, this cell-based assay is costly, technically challenging to perform, and time-consuming. Also, this assay cannot easily measure changes in ATPase activity as a function of drug concentration. We have shown here that measuring ATPase V_{max}/K_m is a simpler way to screen for novel compounds that interfere with drug efflux catalyzed by P-gp.

While this provides a powerful approach to screening for drug−drug interactions, it may not be universally applied to all potential P-gp substrates. For example, stimulation of ATPase activity by certain P-gp substrates such as etoposide and daunorubicin does not display Michaelis−Menten behavior (data not shown). In this case, the inability to accurately define V_{max}/K_m values precludes a careful evaluation by this method. Additionally, we acknowledge that the magnitude of the changes in the catalytic efficiency in ATPase stimulation by doxorubicin, for

example, provides only a qualitative correlation with the change in doxorubicin resistance. Regardless, the speed of collecting data as well as its accuracy in correlating in vitro and ex vivo cellular effects makes this approach an important tool in drug discovery.

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SUPPORTING INFORMATION AVAILABLE

Effects of 5-CHInd on the cytotoxicity of vinblastine, doxorubicin, colchicine, and paclitaxel in KB-V1 cells and parental KB-3-1 cells (Supporting Information 1), data for the effects of 5-CHInd, 5-CEInd, and 5-PhInd on the cytotoxicity of paclitaxel and vinblastine in KB-V1 cells (Supporting Information 2), information about the effects of 5-CHInd on cell viability in KB-V1 and KB-3-1 cells (Supporting Information 3), and structures of 5-CHInd, calcein-AM, cyclosporine A, and paclitaxel (Supporting Information 4). This material is available free of charge via the Internet at http://pubs.acs.org.

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