Modulator-Induced Interference in Functional Cross Talk between the Substrate and the ATP Sites of Human P-glycoprotein†

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ABSTRACT: The human P-glycoprotein (Pgp, ABCB1) is an ATP-dependent efflux pump for structurally unrelated hydrophobic compounds, conferring simultaneous resistance to and restricting bioavailability of several anticancer and antimicrobial agents. Drug transport by Pgp requires a coordinated communication between its substrate binding/translocating pathway (substrate site) and the nucleotide binding domains (NBDs or ATP sites). In this study, we demonstrate that certain thioxanthene-based Pgp modulators, such as *cis*-(*Z*)-flupentixol and its closely related analogues, effectively disrupt molecular cross talk between the substrate, and the ATP, sites without affecting the basic functional aspects of the two domains, such as substrate recognition, binding, and hydrolysis of ATP and dissociation of ADP following ATP hydrolysis. The allosteric modulator *cis*-(*Z*)-flupentixol has no effect on [α-^{32}P]-8-azido-ATP binding to Pgp under nonhydrolytic conditions or on the K_m for ATP during ATP hydrolysis. Both hydrolysis of ATP and vanadate-induced [α-^{32}P]-8-azido-ADP trapping (following [α-^{32}P]-8-azido-ATP breakdown) by Pgp are stimulated by the modulator. However, the ability of Pgp substrates (such as prazosin) to stimulate ATP hydrolysis and facilitate vanadate-induced trapping of [α-^{32}P]-8-azido-ADP is substantially affected in the presence of *cis*-(*Z*)-flupentixol. Substrate recognition by Pgp as determined by [^{125}I]iodoarylazidoprazosin ([^{125}I]IAAP) binding both in the presence and in the absence of ATP is facilitated by the modulator, whereas substrate dissociation in response to vanadate trapping is considerably affected in its presence. In the Pgp F983A mutant, which is impaired in modulation by *cis*-(*Z*)-flupentixol, the modulator has a minimal effect on substrate-stimulated ATP hydrolysis as well as on substrate dissociation coupled to vanadate trapping. Finally, *cis*-(*Z*)-flupentixol has no effect on dissociation of [α-^{32}P]-8-azido-ADP (or ADP) from vanadate-trapped Pgp, which is essential for subsequent rounds of ATP hydrolysis. Taken together, our results demonstrate a distinct mechanism of Pgp modulation that involves allosteric disruption of molecular cross talk between the substrate, and the ATP, sites without any direct interference with their individual functions.

The human multidrug transporter P-glycoprotein (Pgp)[1] is an ATP-dependent efflux pump for a variety of structurally unrelated hydrophobic compounds, ranging from cellular metabolites (such as peptides, phospholipids, and steroids) to antimicrobial (HIV protease inhibitors) and anticancer (taxol) agents (*1*−*3*). It is a member of the ATP binding cassette (ABC) superfamily (*4*, *5*) that includes several clinically important proteins including the cystic fibrosis transmembrane conductance regulator (or CFTR) and the sulfonylurea receptor (SUR) (*6*). Pgp is a 1280 amino acid integral membrane protein with two homologous halves (NH_2-terminal and COOH-terminal), each containing a highly hydrophobic membrane-embedded region and a consensus ATP binding/hydrolysis site, also called the nucleotide binding domain (or NBD) (*7*). The two halves of the protein are connected to each other by an 80 amino acid linker region (*7*) with multiple potential phosphorylation sites of unknown functional significance (*1*, *8*, *9*).

The hydrophobic domain of each half contains six putative transmembrane (TM) α-helices that orient themselves in association with the TMs of the other half to form a single drug translocating pathway (*10*, *11*), in which TMs 4, 5, 6, 10, 11, and 12 are believed to be the major contributors in drug binding and translocation (*12*). Among these, TMs 6 and 12 are arranged in close proximity (*13*) and undergo changes in their relative orientation during ATP hydrolysis (*14*). Pgp possesses an intrinsic ATPase activity that is stimulated by its interaction with drug substrate (*15*). Both ATP sites of Pgp are catalytically active and necessary for its drug transport function (*16*−*19*). The two ATP sites together constitute the catalytic domain of the transporter responsible for energy transduction (*20*, *21*). Although the functional significance of two ATP sites remains controversial (*22*, *23*), experimental evidence suggests that ATP

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[1] Abbreviations: Pgp, P-glycoprotein; [^{125}I]IAAP, [^{125}I]iodoarylazidoprazosin; [α-^{32}P]-8N_3ATP, [α-^{32}P]-8-azido-ATP; ATP, adenosine triphosphate; ADP, adenosine diphosphate; TM, transmembrane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Cis(*Z*), *cis*-(*Z*)-flupentixol; CsA, cyclosporin A; V_i, vanadate; F983A, Pgp F983A mutant; EGTA, ethylene glycol bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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hydrolysis by one drives substrate translocation (*24*, *25*) and dissociation, while the catalytic activity of the other is coupled to resetting the protein conformation for the subsequent round of transport (*23*). ATP-dependent transport by Pgp involves an intimate coordination between the functional states of the ATP sites and the drug binding domain (*26*), as manifested by experimentally detectable conformational changes during its catalytic turnover (*14*, *27*−*29*).

The drug transport function of Pgp has a profound clinical consequence (*2*). It is expressed in 50% of all multidrug-resistant malignant tumors and found to be normally present in vital locations of several healthy tissues altering pharmacokinetics of therapeutic agents that are substrates of the pump (*2*). Although a number of pharmacological agents, called modulators or reversing agents, have been identified that block drug transport by Pgp (*30*, *31*), there remains a growing need for compounds with higher specificity and efficacy (*3*). A major impediment in developing such agents is lack of proper understanding of the modes of action and sites of interaction of Pgp modulators. It is apparent that a more strategic approach to develop clinically useful modulators will require a clear knowledge of the mechanism of action of Pgp and the modes by which its function can be inhibited.

Some of the Pgp modulators, such as verapamil and cyclosporin A (*32*) (first generation inhibitors), are themselves substrates of the pump and inhibit Pgp-mediated drug transport in a competitive manner without interrupting the catalytic cycle (*33*−*37*). However, for the most recently developed second and third generation inhibitors, the modes of action are yet to be determined. Based on experimental evidence, there is a growing consensus in favor of an allosteric mode of action for some of these Pgp inhibitors. Independent studies, including our own (*24*, *38*, *39*), suggest the existence of distinct modulator site(s) within Pgp that are linked to substrate site(s) either by negative or by positive heterotropic cooperativity (*40*−*43*). Although these studies provide useful insights into allosteric modulation of Pgp, the sites of modulator interaction and their influence on the individual catalytic events of Pgp remained poorly understood.

In a recent report using a cell-based assay, we have demonstrated that certain thioxanthene-based Pgp modulators, such as *cis*-(*Z*)-flupentixol and its structurally related analogues, inhibit Pgp-mediated drug transport through interaction at a site distinct from the site of substrate recognition (*39*). Modulation involved a distinct conformational change resulting in formation of a stable but reversible Pgp−substrate complex, retarding substrate dissociation from the transporter. However, the exact mechanism behind this phenomenon remained to be resolved.

During drug transport, the substrate, and ATP, sites of Pgp undergo a series of coordinated changes in their functional states required for ATP-driven substrate translocation (*44*, *45*). Using vanadate, a transition state analogue of ATP hydrolysis, and defined experimental conditions, the major catalytic intermediates of Pgp can be effectively studied. The formation of these catalytic intermediates marked by their specific preferences for transport substrates and nucleotide (ATP or ADP) molecules has been experimentally established (*23*−*25*, *45*). In this report, we systematically evaluate the effect of the allosteric modulator *cis*-(*Z*)-flupentixol on the transitions from one intermediate conformation to the next during a single event of ATP hydrolysis. The substrate binding property of Pgp during these events was determined by studying interactions with the Pgp substrate [^{125}I]IAAP, whereas nucleotide binding to the catalytic domain was determined using a photoaffinity nucleotide analogue [α-^{32}P]-8-azido-ATP.

The results indicate that the allosteric modulator *cis*-(*Z*)-flupentixol, which by itself has no inhibitory effect on substrate recognition, binding, and hydrolysis of ATP, as well as dissociation of ADP, effectively interferes with substrate-stimulated ATP hydrolysis and subsequent dissociation of the substrate molecule that is otherwise coupled to the hydrolytic event.

EXPERIMENTAL PROCEDURES

Chemicals and Cell Lines. Trichoplusia ni (High Five or HF) cells were obtained from Invitrogen (San Diego, CA), and ExCell 400 insect cell medium was from JRH Biosciences (Lexena, KS). Media supplement, L-glutamine, and antibiotic-antimycotic were from BRL Life Technologies (Grand Island, NY). *cis*-(*Z*)-Flupentixol was from Research Biochemicals International. *cis*-(*Z*)-Clopentixol and *cis*-(*Z*)-753 were generous gifts from Dr. James M. Ford, Stanford University School of Medicine, Stanford, CA. Cyclosporin A was purchased from Calbiochem. [^{125}I]Iodoarylazidoprazosin or [^{125}I]IAAP (2200 Ci/mmol) was purchased from NEN PerkinElmer Life Sciences. [α-^{32}P]-8N_3ATP (8.8−13.5 Ci/mmol) was purchased from Affinity Labeling Technologies, Inc. Nonradioactive 8-azido-ATP was purchased from ICN. All other chemicals were obtained from either Sigma or Bio-Rad.

Baculovirus-Mediated Expression of Human Pgp. A recombinant baculovirus harboring the human *MDR*1 cDNA, with a 6× His tag at the C-terminal end, BV-MDR1-(H_6) (*25*), was used to infect High Five insect cells grown in serum-free Excell 400 medium as described (*46*). The recombinant baculovirus containing the Pgp mutant F983A [BV-MDR1-(H_6)-F983A] was constructed in the same way as BV-MDR1-(H_6) (*25*) using recombinant plasmid pBacPakMDR1-(H_6)-F983A. The *Nde*I−*Xho*I fragment of pTM1-MDR1-(H_6)-F983A (containing the codon for amino acid phenylalanine 983 changed to that of alanine) (*47*) was cloned into the wild-type *MDR*1 coding region of plasmid pBacPak9-MDR1-(H_6) to generate pBacPakMDR1-(H_6)-F983A. The recombinant baculovirus BV-MDR1(H_6)-F983A was generated according to ref *25*. For expression of the wild-type Pgp and the mutant F983A, cells were propagated to 80% confluency at 27 °C in the monolayer and infected with the recombinant baculoviruses with a multiplicity of infection of 10 and harvested after 72 h of infection.

Isolation of Crude Membranes from High Five Insect Cells. Crude (cellular) membranes were prepared according to Sarkadi et al. (*48*) with minor modifications (*24*). Briefly, infected cells were harvested and washed twice in phosphate-buffered saline (PBS) containing 1% aprotinin. Washed cells were incubated on ice for 45 min in homogenization buffer [50 mM Tris-HCl, pH 7.5, 50 mM mannitol, 2 mM EGTA,

1 mM DTT, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), and 1% aprotinin] and disrupted by repeated strokes of a Dounce homogenizer. After homogenization, undisrupted cells and nuclei were removed by centrifugation at 500*g* for 20 min. The supernatant was collected and diluted with resuspension buffer (containing 50 mM Tris-HCl, pH 7.5, 300 mM mannitol, 1 mM EGTA, 1 mM DTT, 1 mM AEBSF, and 1% aprotinin) and centrifuged at 100000*g* for 1 h. The pellet was washed once with the same buffer and resuspended in resuspension buffer containing 10% glycerol by passing through a bent hypodermic needle (gauge size 19 and then 23). Membranes, containing predominantly (80−85%) inside-out vesicles (*49*), were stored at −70 °C in aliquots. Protein concentration was measured by a modified Lowry method (*50*) using BSA as a standard.

Photoaffinity Labeling of Pgp with [^{125}I]IAAP. Photoaffinity labeling of crude membranes with the Pgp substrate [^{125}I]IAAP was carried out according to Dey et al. (*24*) with slight modification. Insect cell membranes (15 μg of protein) expressing human Pgp were incubated at room temperature for 10 min under subdued light with 5 nM [^{125}I]IAAP in either 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 300 mM mannitol, and 1% aprotinin (labeling buffer) or 50 mM MES, pH 7.0, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, and 10 mM $MgCl_2$ (ATPase assay buffer), as indicated. Following incubation, membranes were exposed to UV illumination at 365 nm (General Electric F15T8-BLB) for 10 min at room temperature. Following UV cross-linking, 5× SDS−PAGE sample buffer was added to the reaction mixture, which was held at room temperature (~23 °C) for another 30 min, and mixed well before analysis by SDS−PAGE. Where indicated, membranes were preincubated for 5 min with modulators of Pgp prior to the addition of [^{125}I]-IAAP. To study the effect of ATP, preincubation with indicated concentrations of the nucleotide was carried out at 37 °C.

Binding of [α-^{32}P]-8-Azido-ATP to Pgp. Isolated insect cell membranes (0.1 mg of protein) were incubated in ATPase assay buffer (50 mM MES, pH 7.0, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, and 10 mM $MgCl_2$) containing 10 μM [α-^{32}P]-8-azido-ATP (8−13.5 Ci/mmol), on ice for 10 min, prior to UV illumination at 365 nm on ice for 10 min. Wherever indicated, varying concentrations of Pgp modulators were added during incubation with ATPase assay buffer prior to UV illumination. Following photo-cross-linking, samples were resolved by SDS−PAGE and exposed to an X-ray film, and the radioactivity associated with the Pgp band was estimated by PhosphorImager analysis.

Vanadate-Induced 8-Azido-ADP or [α-^{32}P]-8-Azido-ADP Trapping of Pgp. Isolated insect cell membranes (1 mg/mL of protein) were incubated in ATPase assay buffer (50 mM MES, pH 7.0, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, and 10 mM $MgCl_2$) containing 50 μM [α-^{32}P]-8-azido-ATP and 0.25 mM sodium orthovanadate at 37 °C under subdued light for varying periods of time. Reactions (vanadate trapping) were stopped by adding 12.5 mM ice-cold ATP and transferring the tubes immediately on ice. Samples were exposed to UV illumination (365 nm) for 30 min on ice for photo-cross-linking of bound nucleotide. Photo-cross-linked samples were resolved by SDS−PAGE. Gels were dried and exposed to Bio-Max MR film (Kodak) at −70 °C overnight. The radioactivity incorporated into the Pgp band was quantified by using the STORM 860 PhosphorImager system and the software IMAGEQUANT.

For studying [^{125}I]IAAP binding to vanadate-trapped Pgp, isolated membranes were incubated in a similar fashion with nonradioactive 8-azido-ATP (1.25 mM) instead of [α-^{32}P]-8-azido-ATP. After the reaction was stopped by adding 12.5 mM ice-cold ATP and transferring the tube to ice, 5 nM [^{125}I]IAAP was added to each tube and incubated on ice for an additional 10 min period. Wherever indicated, Pgp modulators were added 5 min prior to initiation of trapping with sodium orthovanadate. The samples were photo-cross-linked on ice for 10 min by UV illumination at 365 nm and resolved by SDS−PAGE. Radioactivity associated with the Pgp band was quantified directly by PhosphorImager analysis as indicated and captured on an X-ray film for documentation.

Dissociation of [α-^{32}P]-8-Azido-ADP from Pgp. Pgp in isolated insect cell membranes (1 mg/mL of protein) was vanadate-trapped in the presence of 50 μM [α-^{32}P]-8-azido-ATP, as indicated. Following trapping, free nucleotides and sodium orthovanadate were removed by centrifugation at 200000*g* for 15 min at 4 °C, and the pellet was resuspended in 50 mM MES, pH 7.0, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, and 2 mM DTT. The resuspended membranes were incubated at 37 °C, and at various times 100 μL aliquots were transferred to ice and UV photo-cross-linked at 365 nm for 10 min. Wherever indicated, 0.25 mM sodium orthovanadate, 5 mM 8-azido-ADP, or the indicated concentration of Pgp modulator was added to the samples prior to incubating the resuspended pellet at 37 °C. The photo-cross-linked samples were resolved by SDS−PAGE, and the radioactivity associated with the Pgp band was documented and quantified.

Measurement of ATP Hydrolysis. Pgp-associated ATP hydrolysis in isolated membranes was determined by measuring the vanadate-sensitive release of inorganic phosphate from MgATP in the presence and absence of 0.25 mM sodium orthovanadate using a colorimetric assay originally described by Sarkadi et al. (*48*), with minor modifications (*24*). Inclusion of sodium azide, EGTA, and ouabain in the assay buffer ensured inhibition of other membrane ATPases. While determining K_m values for ATP in the presence of *cis*-(*Z*)-flupentixol and ATPγS, the absorbance (at 880 nm) values for the free phosphate contaminant in the ATP solution were not subtracted.

Data Analysis. The rate constants for [^{125}I]IAAP or [α-^{32}P]-8-azido-ADP dissociation from Pgp were determined by nonlinear regression (in the GraphPad PRISM program) using a first-order rate equation $[L]_t = [L]_0 e^{-kt}$, where $[L]_t$ and $[L]_0$ denote the amounts of ligand bound at times t and 0 min, respectively, and k represents the rate constant of ligand release from Pgp. The values for $t_{1/2}$ (time for half-maximal release) were calculated from the relationship $t_{1/2} = 0.69/k$. For determining the K_m and V_{max} of Pgp ATPase activity, the Henri−Michaelis−Menten equation $v = (V_{max}[S])/(K_m + [S])$ was used in the same program, GraphPad PRISM.

SDS−PAGE and Immunoblot Analysis. Electrophoresis and immunoblot analysis were performed as described previously (*8*).

RESULTS

Modulation by cis-(Z)-Flupentixol Affects neither Substrate Recognition nor ATP Binding. The proper recognition of drug substrate, as well as binding and hydrolysis of ATP, is essential for Pgp-mediated drug transport. Blocking any of these three functional events would lead to failure of the transport process. Using isolated membrane vesicles from High Five insect cells expressing human Pgp, we investigated the effect of the allosteric modulator *cis*-(*Z*)-flupentixol on each of these catalytic steps, in isolation from the others. The effect on substrate binding was determined both in the presence and in the absence of added ATP, and the influence on ATP binding was studied under nonhydrolytic as well as hydrolytic conditions.

Both prazosin and its photoaffinity analogue [^{125}I]iodoaryl-azidoprazosin (or [^{125}I]IAAP) (*51*) are transport substrates of Pgp (*24*, *39*). Since, in the cellular environment, substrate recognition occurs in the presence of ATP and because ATP binding is absolutely essential for Pgp-mediated drug transport, we investigated the effect of *cis*-(*Z*)-flupentixol on [^{125}I]-IAAP binding to Pgp in the presence as well as in the absence of ATP. Pgp, in isolated insect cell membranes, was incubated with 5 nM [^{125}I]IAAP in the presence and absence of 25 μM *cis*-(*Z*)-flupentixol at varying concentrations of ATP (0−2.5 mM) and photo-cross-linked by UV irradiation, as described under Experimental Procedures. Consistent with our previous findings in intact cells and in isolated membranes (*24*, *39*), [^{125}I]IAAP binding to Pgp was stimulated by *cis*-(*Z*)-flupentixol (Figure 1A). A similar stimulation has been observed with the structurally related compounds, *cis*-(*Z*)-clopentixol and *cis*-(*Z*)-753 (data not shown) in intact cells. Interestingly, while ATP had no effect on the basal level (in absence of the modulator) of [^{125}I]IAAP binding to Pgp, stimulation by *cis*-(*Z*)-flupentixol of [^{125}I]IAAP binding was increased 1.8-fold at ATP concentrations of 0.5 mM and higher (Figure 1A). This indicated that although ATP does not have any effect on substrate binding to Pgp, it clearly facilitates *cis*-(*Z*)-flupentixol-induced stabilization of the Pgp−substrate complex.

Pgp modulators that compete with substrate recognition, in general, do not interfere with ATP binding to Pgp. However, some modulators such as flavonoids inhibit interaction of ATP with Pgp, perhaps through direct competition (*52*). Since the *cis*-(*Z*)-flupentixol interaction site does not physically overlap with the substrate site (*24*, *38*), yet inhibits substrate translocation (*39*), we investigated its effect on ATP binding to Pgp. [α-^{32}P]-8-Azido-ATP is a hydrolyzable ATP analogue reliably used for studying nucleotide binding properties of ABC transporters, which has been instrumental in dissecting the catalytic cycle of Pgp. To study the effect of the allosteric modulator *cis*-(*Z*)-flupentixol on ATP binding, [α-^{32}P]-8-azido-ATP interaction with Pgp was studied under nonhydrolyzable conditions, as mentioned under Experimental Procedures. Isolated insect cell membranes were incubated at 0 °C with 10 μM [α-^{32}P]-8-azido-ATP and photo-cross-linked by UV irradiation. In the absence of any modulator or inhibitors, a detectable amount of [α-^{32}P]-8-azido-ATP binding to Pgp was observed (Figure 1B, left panel); this was completely inhibited by 5 mM ATP or 1.25 mM 8-azido-ATP (nonradioactive) (Figure

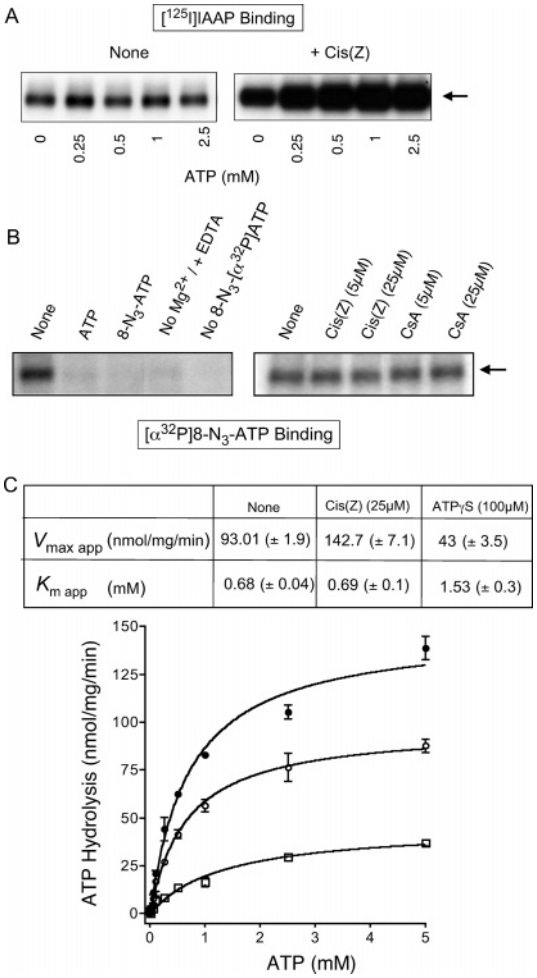

FIGURE 1: Effect of *cis*-(*Z*)-flupentixol on substrate recognition and ATP binding by Pgp. (A) Modulatory effect of *cis*-(*Z*)-flupentixol on [^{125}I]IAAP binding in the presence and absence of ATP. Isolated membranes from High Five insect cells, infected with recombinant baculovirus BV-MDR1(H_6), were photoaffinity labeled with 5 nM [^{125}I]IAAP in the presence (right panel) and absence (left panel) of 25 μM *cis*-(*Z*)-flupentixol with varying concentrations (0−2.5 mM) of ATP in the assay, as described under Experimental Procedures. Following labeling, Pgp in membrane samples (4 μg of protein per well) was resolved by SDS−PAGE, and the radioactivity associated with Pgp was detected in an autoradiogram and quantified using a PhosphorImager (data not shown). (B) Effect of *cis*-(*Z*)-flupentixol on [α-^{32}P]-8-azido-ATP binding to Pgp. Pgp in isolated membranes was photoaffinity labeled with 10 μM [α-^{32}P]-8-azido-ATP under nonhydrolytic conditions (on ice) in the presence and absence of 5 mM ATP (lane 2, left panel), 1.25 mM 8-azido-ATP (lane 3, left panel), 0.25 mM EDTA (with no added Mg^{2+}) (lane 4, left panel), 5 and 25 μM *cis*-(*Z*)-flupentixol [Cis(*Z*)] (lanes 2 and 3, respectively, right panel), and 5 and 25 μM cyclosporin A (CsA) (lanes 4 and 5, respectively, right panel). Samples in lane 5 of the left panel did not have any [α-^{32}P]-8-azido-ATP added in the reaction (background). Samples (32 μg of protein per well) were resolved by SDS−PAGE, and the radioactivity associated with Pgp was detected in an autoradiogram. (C) Effect of *cis*-(*Z*)-flupentixol and ATPγS on the K_m for ATP during ATP hydrolysis by Pgp. The vanadate-sensitive ATP hydrolysis by Pgp was measured in isolated membrane vesicles (see Experimental Procedures) at varying concentrations of ATP in the absence (○) or presence of either 25 μM *cis*-(*Z*)-flupentixol (●) or 0.1 mM ATPγS (a nonhydrolyzable ATP analogue) (□). The data are expressed as nanomoles of ATP hydrolyzed per milligram of protein per minute (nmol mg^{-1} min^{-1}) as a function of ATP concentration and analyzed by nonlinear regression using the computer program GraphPad PRISM 4. To avoid complicacy, the absorbance values for the free phosphate contaminant in the ATP solution were not subtracted. The data presented are the average of two similar experiments.

1B, left), demonstrating the specificity of the interaction. Under similar experimental conditions, neither *cis*-(*Z*)-flupentixol (up to 25 μM) nor cyclosporin A (up to 25 μM) had any effect on [α-^{32}P]-8-azido-ATP binding to Pgp (Figure 1B, right). Exclusion of Mg^{2+} from the reaction and chelating any residual Mg^{2+} by including 0.25 mM EDTA in the assay completely prevented interaction of [α-^{32}P]-8-azido-ATP with Pgp (Figure 1B, left), indicating that the assay measures a functionally relevant ATP binding.

In an alternative approach, we determined the effect of the modulator on the K_m for ATP binding to Pgp. The initial rate of vanadate-sensitive ATP hydrolysis by Pgp was measured at varying concentrations of ATP (from 0 to 5 mM) in the presence and absence of 25 μM *cis*-(*Z*)-flupentixol. Although, *cis*-(*Z*)-flupentixol increased the maximal velocity (the V_{max}) of hydrolysis, no apparent effect on the K_m for ATP was observed (Figure 1C). In contrast, under similar experimental conditions, 0.1 mM ATPγS, which directly competes for the ATP site(s), increased the apparent K_m by more than 2-fold (Figure 1C). Therefore, the data indicated that the allosteric modulator *cis*-(*Z*)-flupentixol does not alter the affinity of Pgp for its nucleotide substrate ATP, even under hydrolytic conditions.

cis-(Z)-Flupentixol Facilitates ATP Hydrolysis but Affects Substrate Dissociation That Is Otherwise Coupled to the Hydrolytic Event. Translocation and dissociation of Pgp-bound substrates are directly coupled to hydrolysis of ATP by the catalytic domain (*20*). This is evident from the fact that vanadate-induced trapping of ADP (or 8-azido-ADP) in the catalytic site, following ATP (or 8-azido-ATP) hydrolysis, results in substantial reduction in the affinity of Pgp for transport substrates, leading to dissociation of the latter (*25*). Vanadate, a phosphate analogue, replaces phosphate from the catalytic site of Pgp formed during ATP hydrolysis (*53*). Since vanadate has an extremely low rate of dissociation, it stabilizes Pgp in a conformation with ADP−vanadate bound in place of ADP−phosphate, following a single event of ATP hydrolysis (*22*, *44*). The trapped state mimics a catalytic intermediate conformation of Pgp that immediately follows the first ATP hydrolytic event. Using [α-^{32}P]-8-azido-ATP (instead of ATP), it is possible to quantify the amount of [α-^{32}P]-8-azido-ADP trapped as a direct measure of the rate and extent of ATP hydrolysis (*54*). On the other hand, replacing [α-^{32}P]-8-azido-ATP with its nonradioactive analogue 8-azido-ATP and including the photoactivatable substrate analogue [^{125}I]IAAP in the assay, it is possible to evaluate the functional status of the substrate binding site during trapping (*23*−*25*). Since *cis*-(*Z*)-flupentixol did not inhibit substrate recognition or ATP binding and hydrolysis, we investigated its effect on the rate of substrate ([^{125}I]IAAP) dissociation in response to vanadate trapping following ATP hydrolysis.

Pgp, in isolated membrane vesicles, was vanadate-trapped with 50 μM [α-^{32}P]-8-azido-ATP and 0.25 mM sodium orthovanadate for varying times (0−10 min) in the presence and absence of 25 μM *cis*-(*Z*)-flupentixol. A time-dependent [α-^{32}P]-8-azido-ADP trapping was observed under both conditions, with a 25% increase in the extent of trapping in the presence of *cis*-(*Z*)-flupentixol (Figure 2A,B). In the presence of the modulator, maximal trapping was achieved within 2.5 min compared to 5 min required in its absence, suggesting no inhibitory effect of *cis*-(*Z*)-flupentixol on [α-^{32}P]-8-azido-ATP hydrolysis and vanadate trapping of [α-^{32}P]-8-azido-ADP.

To study the effect of the modulator on substrate dissociation coupled to ATP hydrolysis, Pgp-containing membranes were vanadate-trapped with 1.25 mM 8-azido-ATP for varying times (0−10 min) in the presence of 5 nM [^{125}I]-IAAP, a photoaffinity analogue of prazosin. Following trapping, Pgp-bound [^{125}I]IAAP molecules were photo-cross-linked, and the radioactivity was detected. In the absence of *cis*-(*Z*)-flupentixol, a time-dependent dissociation of [^{125}I]IAAP was observed with 85% of the bound [^{125}I]IAAP dissociated within the first minute of incubation at 37 °C, and maximal dissociation was achieved within 2.5 min of trapping (k = 2.54 ± 0.87/min, $t_{1/2}$ = 0.27 min) (R^2 = 0.99) (Figure 2C,D). On the other hand, in the presence of 25 μM *cis*-(*Z*)-flupentixol, a considerable delay in [^{125}I]IAAP dissociation was observed, with only around 55% dissociated within 5 min and with maximal dissociation achieved only after 10 min (k = 0.43 ± 0.36/min, $t_{1/2}$ = 1.6 min) (R^2 = 0.83) (Figure 2C,D). This indicated an inhibitory effect of *cis*-(*Z*)-flupentixol on substrate ([^{125}I]IAAP) dissociation from Pgp, which is otherwise tightly coupled to vanadate trapping. When dissociation of bound [^{125}I]IAAP in response to vanadate trapping was studied in the Pgp F983A mutant that is impaired in modulation by *cis*-(*Z*)-flupentixol (*38*, *39*), no such delay in dissociation was observed even in the presence of 25 μM *cis*-(*Z*)-flupentixol (k = 1.86 ± 0.25/min, $t_{1/2}$ = 0.37 min) (R^2 = 0.99) (Figure 2C,D), suggesting that the inhibitory effect on substrate dissociation is dependent on a functional interaction of *cis*-(*Z*)-flupentixol with Pgp. Therefore, the results indicate that while *cis*-(*Z*)-flupentixol does not affect vanadate trapping of ADP following ATP hydrolysis, it considerably interferes with trapping-induced substrate translocation and dissociation.

Impaired Substrate Dissociation Is Due to Interference by cis-(Z)-Flupentixol in Substrate-Stimulated ATP Hydrolysis. There were two distinct possibilities by which *cis*-(*Z*)-flupentixol could affect dissociation of the substrate molecule. One possibility was that *cis*-(*Z*)-flupentixol interfered with the cross talk between the substrate, and the ATP, sites affecting substrate-induced ATP hydrolysis and thus leading to an inadequate driving force for translocating and dissociating the Pgp-bound substrate molecule. The other possibility was an uncoupling effect generated by the modulator disrupting ATP hydrolysis driven conformational change essential for substrate dissociation.

To investigate the effect of *cis*-(*Z*)-flupentixol on substrate-stimulated ATP hydrolysis by Pgp, vanadate-sensitive inorganic phosphate release from MgATP was measured as described (see Experimental Procedures). ATP hydrolysis by Pgp was stimulated by *cis*-(*Z*)-flupentixol in a concentration-dependent manner (Figure 3A, middle). Similarly, the Pgp substrate prazosin (the parent compound of [^{125}I]IAAP) (Figure 3A, left) (*24*) and the competitive modulator verapamil (Figure 3A, right) both stimulated the rate of ATP hydrolysis by Pgp, with maximal stimulation achieved at 100 μM in both cases (data shown up to 50 μM). However, when the rate of ATP hydrolysis was measured in the presence of 50 μM prazosin and increasing concentrations of *cis*-(*Z*)-flupentixol, a concentration-dependent inhibition was observed, with about 50% inhibition occurring at 25 μM *cis*-(*Z*)-flupentixol (Figure 3B, left). At 50 μM *cis*-(*Z*)-

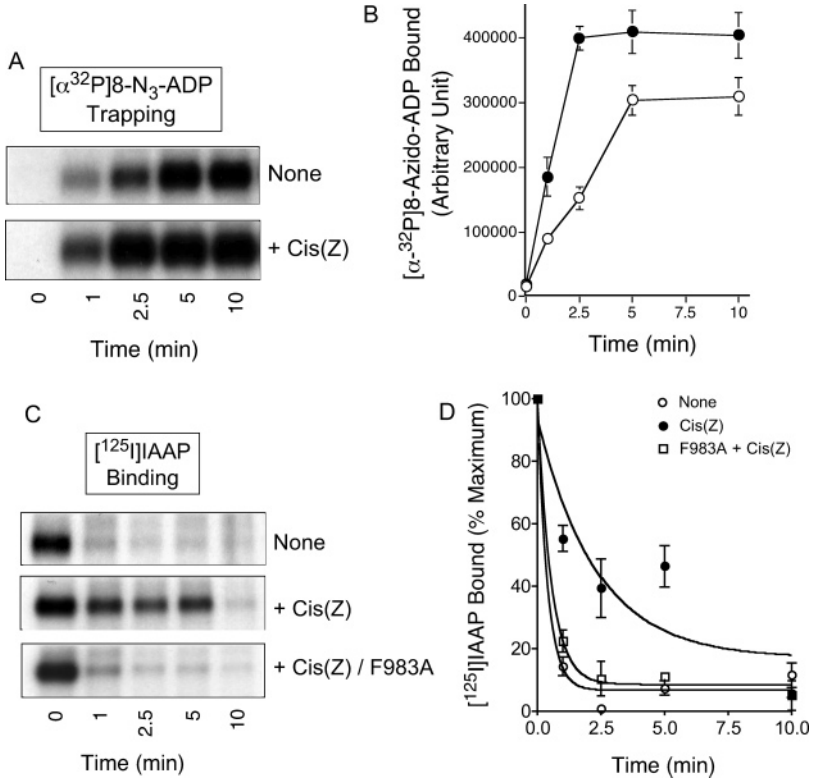

FIGURE 2: Effect of *cis*-(*Z*)-flupentixol on substrate ([^{125}I]IAAP) dissociation coupled to vanadate-induced 8-azido-ADP trapping following 8-azido-ATP hydrolysis. (A, B) Effect of *cis*-(*Z*)-flupentixol on vanadate-induced [α-^{32}P]-8-azido-ADP trapping of Pgp. Pgp in isolated membranes was vanadate-trapped with 50 μM [α-^{32}P]-8-azido-ATP and 0.25 mM sodium orthovanadate in the presence (lower panel) and absence (upper panel) of 25 μM *cis*-(*Z*)-flupentixol for the indicated time, washed to remove unhydrolyzed nucleotides, and UV irradiated to photo-cross-link trapped [α-^{32}P]-8-azido-ADP to Pgp. Samples were resolved by SDS−PAGE, and bound [α-^{32}P]-8-azido-ADP was detected in an autoradiogram (A) and quantified using a PhosphorImager. Data are presented as [α-^{32}P]-8-azido-ADP bound (arbitrary units) to Pgp as a function of time (in minutes) (B). Key: no modulator, ○; +25 μM *cis*-(*Z*)-flupentixol, ●. (C, D) [^{125}I]IAAP binding to vanadate-trapped Pgp. Vanadate trapping of wild-type (top and middle panels) and F983A (bottom panel) Pgp was carried out in the presence (middle and lower panels) and absence (top panel) of 25 μM *cis*-(*Z*)-flupentixol, similar to the above experiment, except that nonradioactive 8-azido-ADP (1.25 mM) was used for trapping, and trapped samples were incubated and photo-cross-linked with 5 nM [^{125}I]IAAP, a Pgp substrate. Samples were resolved by SDS−PAGE, and Pgp-associated radioactivity was detected by autoradiography (C). The radioactivity associated with Pgp was quantified, and the amount of [^{125}I]IAAP bound was expressed as percent maximum bound (at time 0) (D). Key: no modulator, ○; 25 μM *cis*-(*Z*)-flupentixol, ●; 25 μM *cis*-(*Z*)-flupentixol in F983A, □. The data are the average of two similar experiments.

flupentixol, the rate of hydrolysis almost reached that of the basal level, indicating almost complete loss of stimulation by prazosin in the presence of the modulator. In contrast, under similar experimental conditions, prazosin-stimulated ATP hydrolysis was unaffected by the competitive modulator verapamil up to a concentration of 25 μM in the assay (Figure 3B, middle), suggesting that the effect of *cis*-(*Z*)-flupentixol on prazosin-stimulated ATPase activity reflects a specific phenomenon, rather a general effect of two stimulatory drugs synergistically inhibiting the ATPase activity. Consistent with that, stimulation of ATP hydrolysis by 50 μM prazosin in the Pgp F983A mutant was minimally affected by *cis*-(*Z*)-flupentixol (25 μM) (Figure 3B, right), further supporting that the inhibitory effect on substrate-stimulated ATP hydrolysis is mediated through a specific interaction of *cis*-(*Z*)-flupentixol with the allosteric site of Pgp.

cis-(Z)-Flupentixol-Induced Interference of Substrate-Stimulated ATP Hydrolysis Is through Interruption of the Hydrolytic Step. To understand whether inhibition by *cis*-(*Z*)-flupentixol of prazosin-stimulated ATP hydrolysis results from a direct intervention of the ATP hydrolytic step, or whether it is due to interference of a posthydrolytic event preventing multiple catalytic turnovers, we studied the effect of prazosin alone and prazosin in combination with *cis*-(*Z*)-flupentixol on vanadate-induced [α-^{32}P]-8-azido-ADP trapping, following [α-^{32}P]-8-azido-ATP hydrolysis. Vanadate trapping was carried out with 50 μM [α-^{32}P]-8-azido-ATP and 0.25 mM sodium orthovanadate, as mentioned under Experimental Procedures, either in the presence of the Pgp substrate prazosin alone or in the presence of prazosin plus the allosteric modulator *cis*-(*Z*)-flupentixol. In the absence of *cis*-(*Z*)-flupentixol, 25 μM prazosin facilitated [α-^{32}P]-8-azido-ADP incorporation, with a considerable amount bound within 1 min of trapping (Figure 4A, left). In contrast, addition of 25 μM *cis*-(*Z*)-flupentixol to the assay resulted in a 40−60% reduction in trapping of [α-^{32}P]-8-azido-ADP (Figure 4A, right) compared to that in its absence. This suggested that inhibition by *cis*-(*Z*)-flupentixol of prazosin-stimulated ATPase activity was due to an interference with the ATP hydrolytic step rather than in any step following hydrolysis.

cis-(*Z*)-*Flupentixol Has No Effect on ADP Dissociation Following ATP Hydrolysis.* Proper dissociation of ADP and phosphate from the catalytic site of Pgp following ATP hydrolysis is essential for continued turnover of the transporter. Vanadate, a phosphate analogue, inhibits multiple cycles of ATP hydrolysis as well as drug transport by preventing ADP dissociation following the first hydrolytic

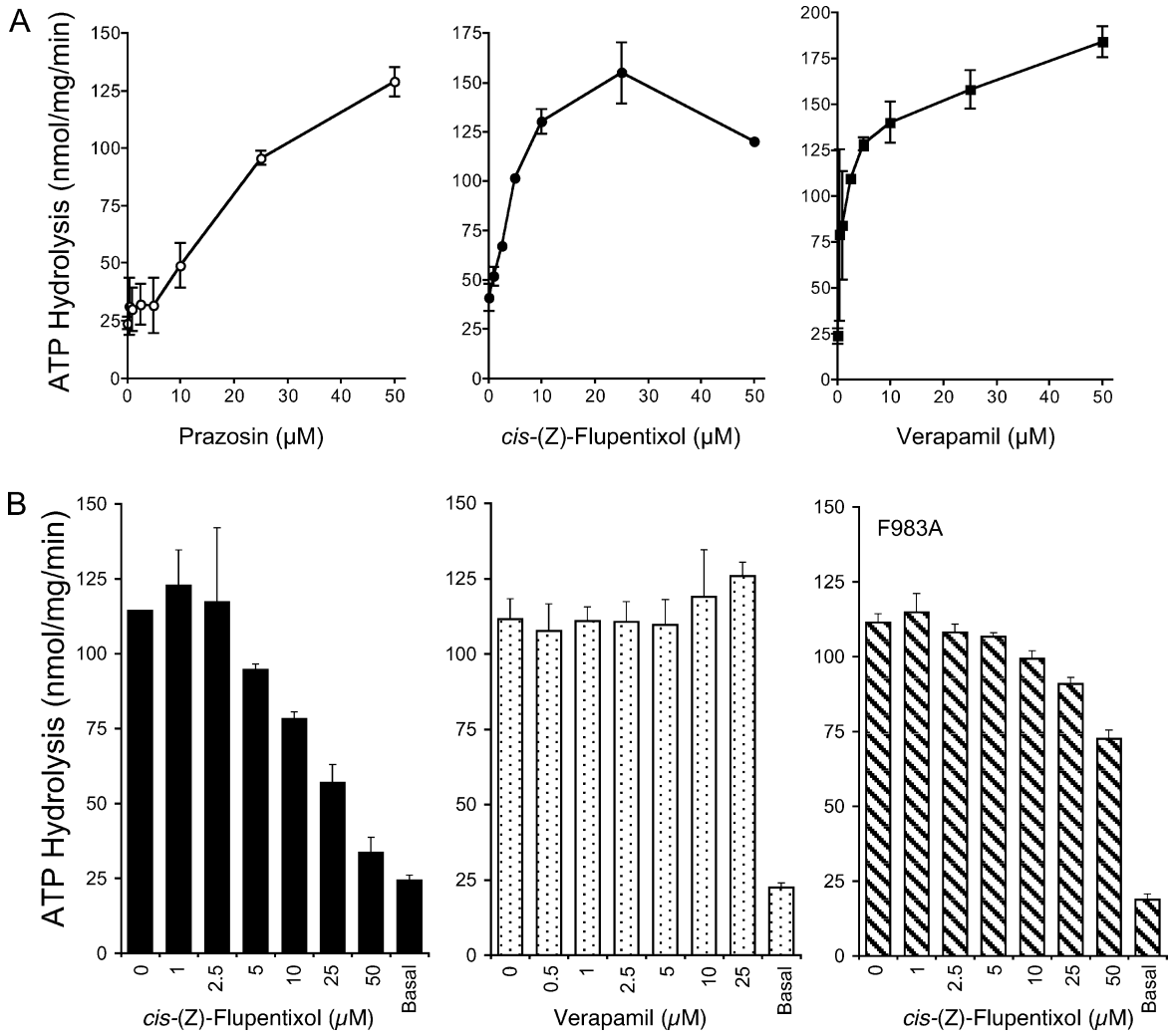

FIGURE 3: Effect of *cis*-(*Z*)-flupentixol on basal and substrate-stimulated ATP hydrolysis. (A) Effect on the basal rate of ATP hydrolysis. The vanadate-sensitive ATP hydrolysis by Pgp was measured in the presence of varying concentrations (0−50 μM) of prazosin (left panel) (○), *cis*-(*Z*)-flupentixol (middle panel) (●), or verapamil (right panel) (■). The data are expressed as nanomoles of ATP hydrolyzed per milligram of protein per minute (nmol mg^{-1} min^{-1}) as a function of substrate/modulator concentration. (B) Effect of modulators on substrate-stimulated ATP hydrolysis. The rate of ATP hydrolysis by the wild-type (left and middle histograms) and F983A (right histogram) Pgp was measured as mentioned in the presence of 50 μM prazosin and varying concentrations (0−50 μM) of the allosteric modulator *cis*-(*Z*)-flupentixol (left and right panels) or the competitive modulator verapamil (middle panel). The data are expressed as nanomoles of ATP hydrolyzed per milligram of protein per minute as a function of modulator concentrations. The data are representative of two similar experiments.

event. To investigate any possible effect of *cis*-(*Z*)-flupentixol on posthydrolytic ADP dissociation, we studied the rate of dissociation of vanadate-trapped [α-^{32}P]-8-azido-ADP from Pgp in the presence and absence of the modulator. Pgp in membrane vesicles was vanadate-trapped in the presence of 50 μM [α-^{32}P]-8-azido-ATP and 0.25 mM sodium orthovanadate, followed by removal of unhydrolyzed nucleotides and incubation at 37 °C in a vanadate- and nucleotide-free medium. The extent of [α-^{32}P]-8-azido-ADP dissociation was studied by photo-cross-linking Pgp-bound [α-^{32}P]-8-azido-ADP after varying time periods. The results indicated that 25 μM *cis*-(*Z*)-flupentixol had no significant effect ($k = 0.09 \pm 0.02$/min, $t_{1/2} = 7.32$ min) ($R^2 = 0.98$) on the rate or extent of [α-^{32}P]-8-azido-ADP dissociation from Pgp ($k = 0.10 \pm 0.02$/min, $t_{1/2} = 6.68$ min) ($R^2 = 0.97$) (Figure 5). Under similar experimental conditions, vanadate alone ($k = 0.037 \pm 0.005$/min, $t_{1/2} = 18.4$ min) ($R^2 = 0.99$) or vanadate plus 8-azido-ADP ($k = 0.033 \pm 0.016$/min, $t_{1/2} = 20.95$ min) ($R^2 = 0.97$) considerably lowered the rate of [α-^{32}P]-8-azido-ADP dissociation, demonstrating the reliability of the assay (Figure 5).

Overall, our data provide convincing evidence in favor of an uncoupling effect of *cis*-(*Z*)-flupentixol on functional cross talk between the substrate, and the ATP, sites of Pgp. Modulation did not affect substrate recognition, ATP binding, ATP hydrolysis, and ADP dissociation, but it effectively interrupted stimulation of ATP hydrolysis by Pgp substrates and subsequently delayed translocation and dissociation of Pgp-bound substrate molecules.

DISCUSSION

Successful pharmacological inactivation of Pgp-mediated drug transport will have a considerable impact on treatment against malignancy and microbial infections. Strategic development of high-efficacy Pgp inhibitors will require detailed knowledge on the mechanism of action of the transporter and the possible modes of its inactivation by small molecules. We have recently demonstrated that the thiox-

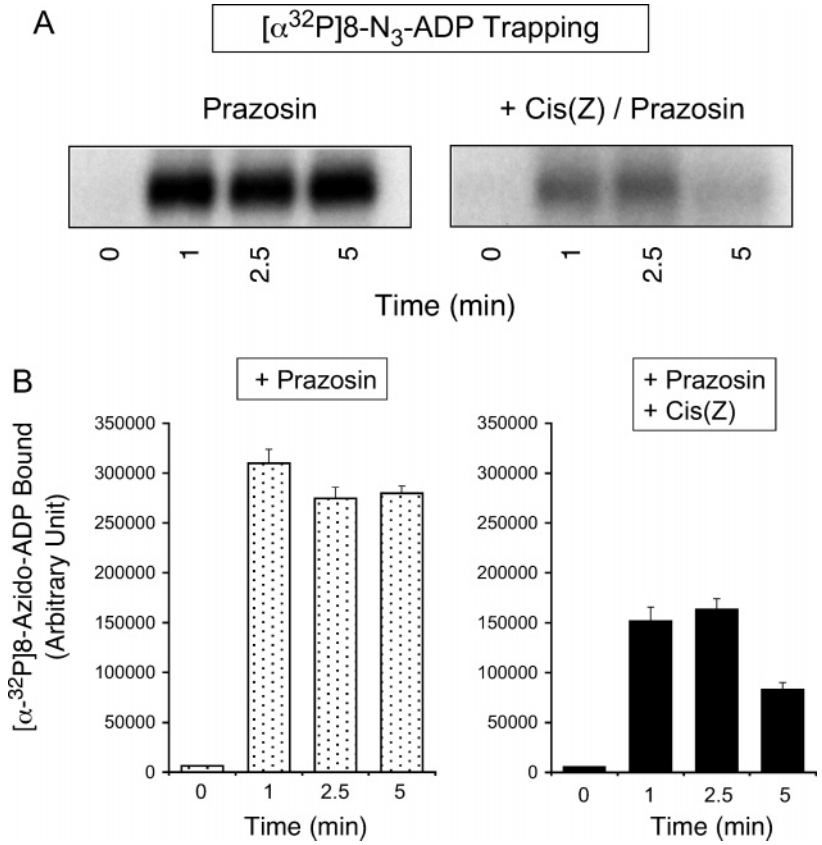

FIGURE 4: Synergistic effect of prazosin and *cis*-(*Z*)-flupentixol on vanadate-induced [α-^{32}P]-8-azido-ADP trapping. (A) Autoradiogram of [α-^{32}P]-8-azido-ADP trapping. Pgp in isolated membranes was vanadate-trapped with 50 μM [α-^{32}P]-8-azido-ATP and 0.25 mM sodium orthovanadate in the presence of either 50 μM prazosin alone (left panel) or 50 μM prazosin + 25 μM *cis*-(*Z*)-flupentixol (right panel), for the indicated times, and washed to remove unhydrolyzed [α-^{32}P]-8-azido-ATP molecules prior to photo-cross-linking the trapped [α-^{32}P]-8-azido-ADP to Pgp by UV irradiation. The samples (32 μg of protein) were resolved by SDS−PAGE, and bound [α-^{32}P]-8-azido-ADP was detected by autoradiography. (B) Quantification of radioactivity associated with Pgp. Radioactivity associated with Pgp in the above experiment was quantified using a PhosphorImager and expressed as [α-^{32}P]-8-azido-ADP trapped (arbitrary units) as a function of time (in minutes). The graph on the left represents [α-^{32}P]-8-azido-ADP binding to Pgp in the presence of prazosin (50 μM) alone and that on the right in the presence of 50 μM prazosin + 25 μM *cis*-(*Z*)-flupentixol. The data are the average of two similar experiments.

anthene-based modulator *cis*-(*Z*)-flupentixol and some of its closely related analogues inhibit drug transport by interacting with Pgp at a site functionally distinct from the site of substrate recognition (*38*, *39*). Unlike competitive inhibitors, such as cyclosporin A, these modulators do not interfere with substrate binding to Pgp, rather they prevent substrate translocation and dissociation (*39*). However, the molecular events leading to inhibition of the translocation step remained to be determined. In this report, by categorically studying the experimentally detectable catalytic events involved in ATP-driven drug transport, we demonstrate that *cis*-(*Z*)-flupentixol specifically interferes with functional cross talk between the substrate and the ATP sites, without affecting other catalytic events, such as substrate recognition, binding and hydrolysis of ATP, or ADP dissociation following hydrolysis.

Our experimental data suggest that modulator interaction at the allosteric site influences the substrate site independent of nucleotide binding (Figure 1A, left); however, the ability of *cis*-(*Z*)-flupentixol to stimulate substrate binding is enhanced by ATP under hydrolytic condition (Figure 1A, right). One possibility is that nucleotide binding facilitates modulator interaction with Pgp, which in turn enhances the stimulatory effect of the latter. Alternatively, it is possible that at any given time a certain percentage of the Pgp molecules remain in a low-affinity conformation, which undergoes a transition upon ATP hydrolysis to a state more compatible to substrate binding. This is consistent with the finding that ATP hydrolytic events of Pgp have two distinct consequences (*23*). While hydrolysis at one site leads to reduced affinity for substrate (*24*, *25*), the subsequent hydrolysis at the other restores normal substrate binding (*23*). Since stimulation by *cis*-(*Z*)-flupentixol is mediated through intervention of the (substrate) dissociation step (*39*), any event that improves chances of Pgp−substrate association is expected to have an additive effect on the level of stimulation.

On the other hand, although *cis*-(*Z*)-flupentixol does not alter ATP binding to Pgp (Figure 1B), it significantly stimulates the rate of hydrolysis (Figure 3A, middle) by the catalytic domain. Since the stimulatory effect on both substrate interaction and ATP hydrolysis is abrogated in the Pgp F983A mutant (*38*), which is impaired in modulation by *cis*-(*Z*)-flupentixol (*38*, *39*), it suggests that both phenomena are mediated through modulator interaction at the allosteric site. Therefore, the data suggest that the allosteric modulator site can communicate directly with the substrate, as well as the ATP, sites, independent, but not necessarily exclusive, of each other. A similar effect on substrate binding and on ATP hydrolysis has been observed in structurally related thioxanthene derivatives, such as *cis*-(*Z*)-clopentixol and *cis*-(*Z*)-753 (data not shown), suggesting a common

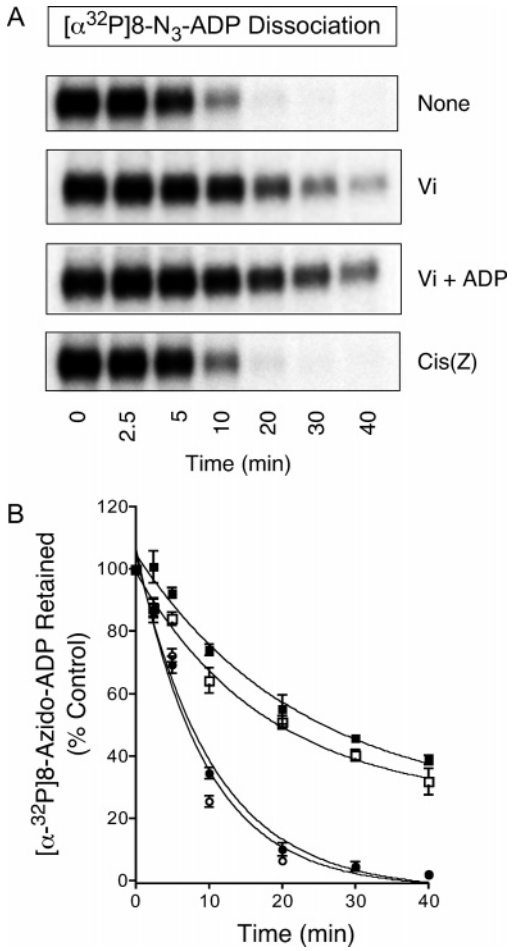

FIGURE 5: Effect of *cis*-(*Z*)-flupentixol on dissociation of trapped [α-^{32}P]-8-azido-ADP. (A) Pgp was vanadate-trapped with 50 μM [α-^{32}P]-8-azido-ATP in the presence of 0.25 mM sodium orthovanadate. Following trapping, membranes were washed to remove unhydrolyzed nucleotides and incubated at 37 °C for varying times in the absence (none, top panel) or in the presence of 0.25 mM sodium orthovanadate (V_i, second panel from the top), 0.25 mM sodium orthovanadate + 5 mM 8-azido-ADP (V_i + ADP, second panel from the bottom), or 25 μM *cis*-(*Z*)-flupentixol [Cis(*Z*), bottom panel]. Following incubation, samples were photo-cross-linked and resolved by SDS−PAGE as described, and Pgp-bound [α-^{32}P]-8-azido-ADP was detected by autoradiography. A total of 32 μg of proteins was loaded per well. (B) Radioactivity associated with Pgp was quantified in a PhosphorImager, and the amount of [α-^{32}P]-8-azido-ADP retained was expressed as the percentage of the amount bound at 0 min. Key: none, ○; vanadate, □; vanadate + 8-azido-ADP, ■; *cis*-(*Z*)-flupentixol, ●.

mechanism of action for these thioxanthene-based modulators. In the present study, for an in-depth analysis, we focused on the compound *cis*-(*Z*)-flupentixol as a representative of this class of modulators.

One interesting observation is that, although *cis*-(*Z*)-flupentixol, by itself, enhances the basal rate of ATP hydrolysis (Figure 3A, middle), it severely affects stimulation of the hydrolytic activity by Pgp substrates, such as prazosin (the parent compound of [^{125}I]IAAP) (Figure 3B, left). This is suggestive of an active interference by the allosteric modulator in the flow of information between the substrate, and the ATP, sites. One assumption is that, since both compounds independently enhance the rate of ATP hydrolysis, the stimulatory signal originating from *cis*-(*Z*)-flupentixol interaction with the allosteric modulator site dominates over the signal generated by prazosin binding to the substrate site. However, since the combined presence of prazosin and *cis*-(*Z*)-flupentixol reduced the rate of ATP hydrolysis (Figure 3B, left) considerably below the level induced by their individual actions (Figure 3A, left and middle), it suggests that the signals originating from the two sites are not mutually exclusive. It also rules out the possibility of inhibition due to physical displacement (direct or indirect) of the bound substrate by the allosteric modulator. The reduced effectiveness of *cis*-(*Z*)-flupentixol to inhibit the prazosin-stimulated ATP hydrolysis (Figure 3B, right) in the Pgp F983A mutant further confirms that the inhibitory action of the modulator is mediated through its interaction at the allosteric site of Pgp and not by its nonproductive (nonstimulating) association with the substrate site, blocking access to prazosin. In contrast, the lack of any synergistic or additive effect of the combined presence of prazosin and verapamil (which also stimulates ATP hydrolysis) (Figure 3B, middle) indicates a mutually exclusive interaction between the substrate (prazosin) and the competitive modulator (verapamil) with Pgp.

The inhibitory effect of *cis*-(*Z*)-flupentixol on prazosin-stimulated ATP hydrolysis could be a consequence of two alternative possibilities. It could either be due to interference with the ATP hydrolytic step or be due to intervention of a posthydrolytic event preventing multiple catalytic turnovers. Results from vanadate trapping of [α-^{32}P]-8-azido-ADP following [α-^{32}P]-8-azido-ATP hydrolysis (Figure 4), and the studies on the rate of [α-^{32}P]-8-azido-ADP dissociation (Figure 5), clearly suggest that the inhibition is at the hydrolytic step. Since substrate translocation and dissociation are coupled to the ATP hydrolytic step and not to ADP dissociation (*24*, *25*), it is expected that inhibition of the former would result in impaired dissociation of the Pgp-bound substrate. Consistent with that, dissociation of Pgp-bound [^{125}I]IAAP (prazosin analogue) that is tightly coupled to vanadate trapping was substantially delayed in the presence of *cis*-(*Z*)-flupentixol (Figure 2C,D). The dissociation of bound [^{125}I]IAAP coupled to vanadate trapping was minimally affected by *cis*-(*Z*)-flupentixol in the Pgp F983A mutant (Figure 2C,D), further emphasizing the involvement of the allosteric modulator site in the phenomenon.

Allosteric communication between the nucleotide binding domains and the substrate sites of Pgp has already been established. This is evident from substrate-stimulated ATP hydrolysis (*15*) and ATP hydrolysis-driven substrate translocation and/or dissociation (*20*, *24*, *25*). Experimentally detectable conformational changes in response to substrate recognition or ATP binding and hydrolysis further support this conclusion (*14*, *55*−*58*). In this study, we demonstrate that the allosteric site of interaction for the thioxanthene-based Pgp modulators directly communicates with the substrate, as well as the ATP, sites, independent of each other. Furthermore, the nature of the modulatory signal changes depending on whether the substrate site is occupied or not, indicating a reciprocal cross talk between the substrate, and the modulator, site. The loss of stimulation (Figure 3B, left) could be an outcome of a negative synergistic effect from both substrate, and modulator, sites being occupied at the same time. In the Pgp F983A mutant, the stimulatory signal (for ATP hydrolysis) originating from the substrate site is minimally affected in the presence of *cis*-(*Z*)-flupentixol (Figure 3B, right); this suggests that the modulator-induced interference in cross talk between the

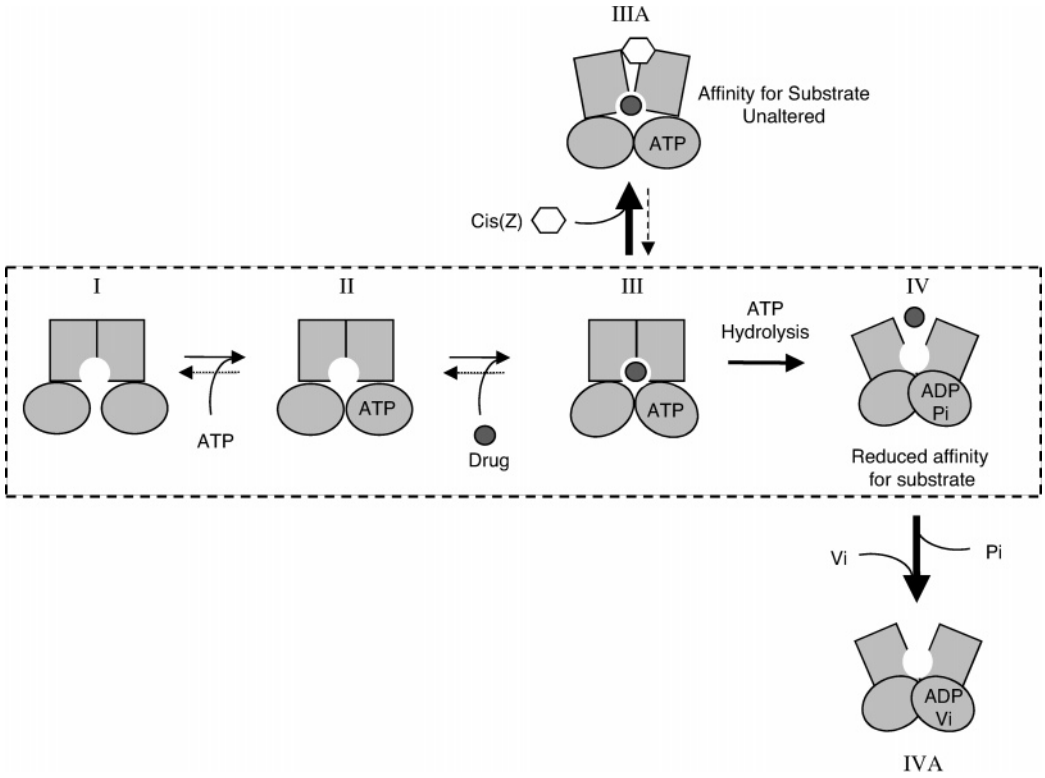

FIGURE 6: Schematic representation of the plausible mode of action of the allosteric modulator *cis*-(*Z*)-flupentixol on Pgp function. In this scheme, the two TM regions and the ATP binding domains are represented by squares and ellipses, respectively. The scheme shows a single event of ATP hydrolysis including substrate recognition and ATP binding. The filled sphere (small) represents transport substrate, and the clear hexagon designates the allosteric modulator *cis*-(*Z*)-flupentixol. The drug binding site of Pgp is shown by an open sphere within the TM domains, and its high- and low-affinity states are represented by closed (inward facing) and open (outward facing) conformations. The Pgp conformations I, II, III, and IV represent the major experimentally detectable catalytic intermediates during the first ATP hydrolytic event. State I has no ligand bound, state II has ATP bound, state III has both substrate and ATP bound, and state IV has ADP•P_i (ADP•phosphate) bound following ATP hydrolysis. Interaction with *cis*-(*Z*)-flupentixol results in formation of a stable but reversible complex between Pgp and its substrate (IIIA), incompatible for ATP hydrolysis and drug transport. State IVA represents the vanadate-trapped conformation of Pgp with the substrate molecule dissociated. The dotted box indicates the catalytic steps leading to a single event of ATP hydrolysis and drug translocation coupled to it. For simplicity, activity of only one ATP site has been shown. The sequence of events leading to the basal ATPase activity in the absence of drug substrate is not included in the scheme.

substrate, and the ATP, sites is generated through specific interaction of *cis*-(*Z*)-flupentixol at the allosteric site and not from elsewhere within the protein.

The fact that *cis*-(*Z*)-flupentixol does not inhibit the basal rate of ATP hydrolysis by Pgp (Figure 3A, middle) but selectively blocks the stimulatory signal generated by substrate binding (Figure 3B, left) has an interesting mechanistic implication. The functional significance of the basal ATPase activity has been a subject of considerable debate and experimental investigations. One possible interpretation is that it could be due to low-level binding and transport of endogenous substrate(s), such as phospholipids (*59*−*62*). Alternatively, the basal activity could represent Pgp undergoing a futile catalytic cycle hydrolyzing ATP without drug translocation (*29*, *63*). Since *cis*-(*Z*)-flupentixol stimulates the basal rate of ATP hydrolysis (Figures 2A, lower, and 3A, middle) but inhibits the substrate-stimulated ATPase activity (Figure 3B, left), it is possible that the two events are mechanistically distinct. A mechanistic difference between the basal and the substrate-stimulated ATPase activity of Pgp has been recently proposed using different experimental approaches (*63*−*65*).

Allosteric modulation of ABC transporters has been a matter of continuing investigation. The cystic fibrosis transmembrane conductance regulator- (CFTR-) mediated ATP hydrolysis and chloride transport activity are directly regulated by the phosphorylation state of its R-domain (*66*). The protein has negligible chloride channel activity until the R-domain is phosphorylated at multiple sites and undergoes a conformational change (*67*). The sulfonylurea receptor (SUR) by itself acts as a modulatory subunit of the ATP-sensitive inwardly rectifying potassium channel $Kir_{6.x}$ (*68*, *69*). Interaction of Mg^{2+} nucleotides with the nucleotide binding domains of SUR mediates activation of the K_{ATP} channel (*70*). The human transporter associated with antigen presenting (TAP) undergoes modulatory regulation by viral peptides as a mechanism of evading the immune system (*71*). Binding of the human cytomegalovirus- (HCMV-) encoded protein, gpUS6, to TAP1 inhibits ATP binding and hydrolysis without any effect on the interaction of the transporter with its peptide substrates (*72*). Interestingly, the interaction site for the gpUS6 peptide is to a luminal domain of the ER-resident TAP1 protein, while the site for ATP binding and hydrolysis is on the cytosolic side, indicating a transmembrane allosteric cross talk between the two domains. Similarly, the herpes simplex virus (HSV-1) protein ICP47 binds to TAP from the cytosolic side, specifically blocking substrate peptide binding and ATP hydrolysis without any effect on nucleotide (ATP or ADP) binding (*73*). Recently, for the human multidrug resistance protein 1 (MRP1), an

interaction with reduced glutathione (GSH) has been shown to induce conformational changes that enhance ATP binding and/or hydrolysis, suggesting an allosteric effect of GSH in coupling ATP hydrolysis to drug transport (*74*).

Our present study demonstrates allosteric modulation of the human Pgp by small molecules through their interaction at a functionally distinct interaction site, inducing impaired cross talk between the substrate, and the ATP, binding domains. Although allosteric modulation of Pgp function has been reported by several groups (*21*), little is known about the molecular mechanism and the sites for modulator interaction. The modulatory effects on Pgp vary from inhibition (*38*−*42*, *75*) to stimulation (*76*−*78*) of drug transport and, in some instances, to alteration of substrate specificity (*79*). Molecular and pharmacological characterization of the allosteric sites will improve the chances of developing high-efficacy inhibitors of the transporter and is also likely to provide insight into its potential functional regulation by endogenous metabolites. It is too premature to predict the spatial relationship of this allosteric modulator site to the drug binding site of Pgp. Nevertheless, the location of residue F983 in TM12 (*10*, *80*) suggests that a crucial interaction point for these modulators lies outside the major substrate binding pocket of Pgp.

In summary, on the basis of the results from our current study and previously published data, we propose a working scheme depicting the mode of action of the allosteric modulator *cis*-(*Z*)-flupentixol and its closely related analogues (Figure 6). According to the model, ATP and drug substrate can bind Pgp independently of each other; however, binding of the latter stimulates ATP hydrolysis. ATP hydrolysis creates a conformational change in Pgp that drives substrate translocation. In the presence of *cis*-(*Z*)-flupentixol, substrate-stimulated ATP hydrolysis is inhibited, and at the same time the Pgp−substrate complex is stabilized (Figure 6, conformation IIIA). This interrupts the normal catalytic turnover of Pgp; consequently, Pgp-mediated drug transport is inhibited. However, when the substrate site remains unoccupied, *cis*-(*Z*)-flupentixol facilitates ATP hydrolysis. It is not yet clear whether the modulator comes off after each cycle of hydrolysis or remains bound to Pgp for multiple cycles. Detailed mapping of the modulator interaction site and the effect of the modulator on the second ATP hydrolytic event are currently under investigation.

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