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Inhibition of Multidrug Resistance-Linked P-Glycoprotein (ABCB1) Function by 5′-Fluorosulfonylbenzoyl 5′-Adenosine: Evidence for an ATP Analogue That Interacts with Both Drug-Substrate-and Nucleotide-Binding Sites

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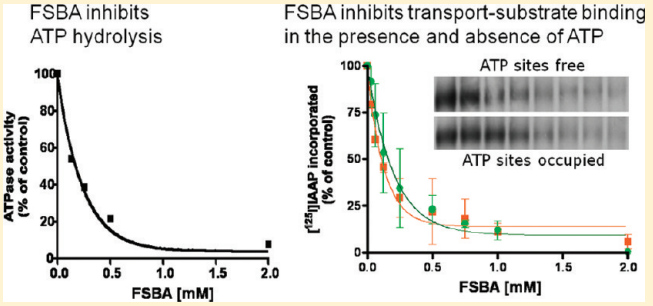
Supporting Information

ABSTRACT: 5′-Fluorosulfonylbenzonyl 5′-adenosine (FSBA) is an ATP analogue that covalently modifies several residues in the nucleotide-binding domains (NBDs) of several ATPases, kinases, and other proteins. P-glycoprotein (P-gp, ABCB1) is a member of the ATP-binding cassette (ABC) transporter superfamily that utilizes energy from ATP hydrolysis for the efflux of amphipathic anticancer agents from cancer cells. We investigated the interactions of FSBA with P-gp to study the catalytic cycle of ATP hydrolysis. Incubation of P-gp with FSBA inhibited ATP hydrolysis (IC_{50} = 0.21 mM) and the binding of 8-azido[α-^{32}P]ATP (IC_{50} = 0.68 mM). In addition, ^{14}C-FSBA cross-links to P-gp, suggesting that FSBA-mediated inhibition of ATP hydrolysis is irreversible due to covalent modification of P-gp. However, when the NBDs were occupied with a saturating concentration of ATP prior to treatment, FSBA stimulated ATP hydrolysis by P-gp. Furthermore, FSBA inhibited the photo-cross-linking of P-gp with [^{125}I]iodoarylazidoprazosin (IAAP; IC_{50} = 0.17 mM). As IAAP is a transport substrate for P-gp, this suggests that FSBA affects not only the NBDs but also the transport-substrate site in the transmembrane domains. Consistent with these results, FSBA blocked efflux of rhodamine 123 from P-gp-expressing cells. Additionally, mass spectrometric analysis identified FSBA cross-links to residues within or nearby the NBDs but not in the transmembrane domains, and docking of FSBA in a homology model of human P-gp NBDs supports the biochemical studies. Thus, FSBA is an ATP analogue that interacts with both the drug-binding and ATP-binding sites of P-gp, but fluorosulfonyl-mediated cross-linking is observed only at the NBDs.



The ATP binding cassette (ABC) family of transport proteins constitutes one of the largest gene families.[1] The clinical importance of this family is apparent from the fact that, of the 48 human ABC transporters, 17 are implicated in human diseases.[2] P-glycoprotein (P-gp), the first human ABC transporter to be discovered,[3] plays a significant role in multidrug resistance (MDR). MDR is a phenomenon in which cells show resistance to chemically diverse drugs with multiple mechanisms of action and is one of the mechanisms by which tumors become resistant to chemotherapeutic drugs.[4] Thus, significant effort has been expended over the past two decades to understand the mechanism of P-gp in particular and ABC proteins in general [for reviews see refs 5−8]. P-gp is an energy-dependent molecular pump that utilizes the energy of ATP hydrolysis to move drug substrates across the cell membrane.[9] The enzymatic hydrolysis of ATP and the transport of drug substrates are tightly coupled phenomena that occur in different domains of the protein.[10] The movement of drug substrates occurs as a consequence of conformational changes in the membrane-spanning transmembrane domains (TMDs); ATP hydrolysis occurs at the intracellular nucleotide-binding domains (NBDs). The development of probes for these two regions of the protein has played a significant role in elucidating the catalytic cycle of P-gp-mediated ATP hydrolysis as well as the mechanism of the transport cycle.

ABC transporters have relatively low affinities (in the micromolar range) for their drug substrates as well as for nucleotides.[11] These low affinities present technical difficulties that have been enumerated in detail by Senior and colleagues.[12] Photoaffinity reagents that can form covalent bonds with the amino acid side chains have proved extremely useful in probing the substrate- and nucleotide-binding

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sites.[13] Over the past few decades there has been a concerted effort to design nucleotide-based affinity labels.[14] The general consensus is that labels that incorporate the reactive group into the 5′-hydroxyl group cause minimal perturbation of the adenosine moiety.[15] The Colman group at the University of Delaware has taken the lead in synthesizing and characterizing such reagents. One of the most successful reagents to emerge from their work has been 5′-fluorosulfonylbenzonyl 5′-adenosine (FSBA).

The sulfonyl fluoride group of FSBA that replaces the phosphate groups of ATP is a reactive functional group that can react with several amino acids.[16−18] As the sulfonyl fluoride participates in a broad range of reactions, there is a reasonable probability of reaction within any particular active site.[15] The sulfonyl fluoride acts as an electrophilic agent that has been shown to form covalent bonds with tyrosine, lysine, histidine, serine, and cysteine.[15] Thus, FSBA has been used to localize the nucleotide-binding sites, characterize the active site, and study the catalytic mechanism of numerous enzymes. These include the cAMP-dependent protein kinase II,[19] isocitrate dehydrogenase kinase,[20] rabbit muscle pyruvate kinase,[21] myosin, multifunctional protein CAD,[22] and the mitochondrial F_1-ATPase.[23] This reagent has proved particularly useful in studying the protein kinase family,[24] and studies show that FSBA produces a stable, isolable product after reacting with the active sites of the enzymes.[25] FSBA−protein interactions have been studied extensively, including the effect of FSBA binding and/or cross-linking on enzyme activity and kinetics,[26,27] the use of antibodies and LC-MS to identify the sites of FSBA cross-linking,[28] and the use of [^{14}C]-labeled FSBA.[29] Nonetheless, there have been no studies that exploit FSBA to probe the nucleotide-binding sites of ABC transporters.

In this study we show that FSBA interacts with both the nucleotide- and substrate-binding sites of P-gp. In addition, our data demonstrate that FSBA is cross-linked to P-gp. Significantly, FSBA also reverses P-gp-mediated transport of a fluorescent substrate in intact cells. These results show that, as may be expected based on the interactions of FSBA with numerous other proteins (for review, see ref 14), this nucleotide analogue interacts with the NBDs of P-gp. In addition, we demonstrate that FSBA interacts with the substrate-binding sites in the TMDs of this transporter. A similar dual effect has also been observed with disulfiram, although the mechanism is significantly different. Disulfiram, which is not an ATP analogue, reacts with the Walker A cysteine residue in each NBD, thus inhibiting ATP hydrolysis, while it also interacts at the substrate-binding sites of P-gp.[30,31] However, in Cys-less P-gp, it interacts only at the transport-substrate site in the TMDs. FSBA, as an ATP analogue, either competes with ATP for binding at the NBDs (even in Cys-less P-gp) or binds to another site and has an effect on ATP binding at NBDs. It also interacts at the drug-binding sites, while ATP does not. Using mass spectrometric analysis, we were able to demonstrate that FSBA cross-links residues in NBDs (for example, K411 in NBD1) as well as in the regions near NBDs. We were not able to detect FSBA cross-linked peptides originating from the TMDs, suggesting that interaction between the FSBA and the substrate-binding site in TMDs is reversible. In addition, this FSBA interaction at the TMDs does not require functional ATP sites, as the IAAP labeling in the Y401A/Y1044A double mutant lacking ATP-binding capacity is still inhibited by FSBA.

MATERIALS AND METHODS

Chemicals. [^{125}I]Iodoarylazidoprazosin ([^{125}I]IAAP, 2200 Ci/mmol) and [adenine-8-^{14}C]FSBA (57 mCi/mmol) were obtained from Perkin-Elmer Life Sciences (Boston, MA). 8-Azido[α-^{32}P]ATP (15−20 Ci/mmol) and 8-azidoATP were purchased from Affinity Labeling Technologies, Inc. (Lexington, KY). The P-gp-specific monoclonal antibody C-219 was obtained from Fujirebio Diagnostics Inc. (Malvern, PA). All other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Cell Lines and Culture Conditions. KB-3-1 and P-gp-overexpressing KB-V1 cells were maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin, and the KB-V1 cells were grown in media containing 1 μg/mL vinblastine.[32]

Preparation of Crude Membranes from High-Five Insect Cells Infected with Recombinant Baculovirus Carrying the Wild-Type and Mutant Human *MDR*1 Gene. High-Five insect cells (Invitrogen, Carlsbad, CA) were infected with the baculovirus carrying the human *MDR*1 cDNA (either wild-type or Y401A/1044A or the cys-less mutant where all seven native cysteine residues are replaced with alanine) with a 6X histidine tag at the C-terminal end as described previously.[33] Crude membranes were prepared and stored at −70 °C as described previously.[33,34]

Purification and Reconstitution of P-gp. Human P-gp from crude membranes of High-Five insect cells was purified as described previously.[33,35] The crude membranes were solubilized with octyl β-D-glucopyranoside (1.25%) in the presence of 20% glycerol and a lipid mixture (0.1%). Solubilized proteins were subjected to metal affinity chromatography (Talon resin, Clontech, Palo Alto, CA) in the presence of 0.95% octyl β-D-glucopyranoside and 0.04% lipid; 80% purified P-gp was eluted with 200 mM imidazole. P-gp in the 200 mM imidazole fraction was then concentrated (Centriprep-50, Amicon, Beverly, MA) to ∼0.5−1.5 mg/mL and stored at −70 °C. P-gp was identified by immunoblot analysis using the monoclonal antibody C219[33] and quantified by the Amido Black protein estimation method as previously described.[36] Purified P-gp was reconstituted into proteoliposomes by dialysis using a lipid:protein ratio of 10:1 as described.[37] The purified Pgp in proteoliposomes exhibited 50 μM verapamil-stimulated ATPase activity in the range of 1100−1300 nmol of P_i/min/mg of protein as previously described.[35,38]

ATPase Assay. Crude membranes (10 μg protein/100 μL) from High-Five cells expressing P-gp were incubated at 37 °C for 30 min with varying concentrations of FSBA in the presence and absence of sodium orthovanadate (Vi) (0.3 mM) in ATPase assay buffer (50 mM MOPS-KOH, pH 7.5, 50 mM KCl, 5 mM sodium azide, 1 mM EGTA, 1 mM ouabain, 10 mM $MgCl_2$). The reaction was initiated by the addition of 5 mM ATP and incubated for 20 min at 37 °C. SDS solution (0.1 mL of 5% SDS) was added to terminate the reaction, and the amount of inorganic phosphate released was quantified with a colorimetric reaction, as described previously.[11]

Photo-Cross-Linking of 8-Azido[α-^{32}P]ATP to P-gp. Crude membranes of High-Five insect cells (50−100 μg of protein) or purified and reconstituted protein (5−10 μg of protein) were incubated in an ATPase assay buffer (50 mM MES-Tris, pH 6.8, 50 mM KCl, 5 mM sodium azide, 1 mM EGTA, 1 mM ouabain and 10 mM $MgCl_2$) containing 10 μM [α-^{32}P]8azidoATP (10 μCi/nmol) in the dark at 4 °C for 5 min. The samples were irradiated with a UV lamp assembly (PGC Scientifics, Gaithersburg, MD) fitted with two black light (self-filtering) UV-A long wave F15T8BLB tubes (365 nm) for 10 min on ice (4 °C). Ice-cold ATP (10 mM) was added to displace excess noncovalently bound 8-azido[α-^{32}P]ATP. After SDS-PAGE on a 7% NuPAGE gel, the gels were dried and exposed to Bio-Max MR film (Eastman Kodak Co.) at −70 °C for 12−24 h. The radioactivity incorporated into the P-gp band was quantified using the STORM 860 PhosphorImager system

(Molecular Dynamics, Sunnyvale, CA) and the software ImageQuaNT. One phase decay mode was used to fit the data given in Figure S2 (additional details are given in the legend to Figure S2).

Photoaffinity Labeling with [^{125}I]IAAP. The crude membranes of High-Five insect cells (60−75 μg protein/100 μL) were incubated at room temperature in ATPase buffer with [^{125}I]IAAP (5−7 nM) for 5 min under subdued light. The samples were photo-cross-linked for 5 min at room temperature followed by electrophoresis and quantification as described previously.[34] Modifications to this procedure in specific experiments are detailed in the figure legends.

Chemical Cross-Linking of P-gp with [^{14}C]FSBA. Purified and reconstituted P-gp (15−40 μg protein) was incubated with 100 μM [^{14}C]FSBA for 30 min at 37 °C. The reaction was stopped by the addition of SDS-PAGE loading buffer, and the samples were electrophoresed on a 7% NuPage gel at constant voltage. The dried gel was used to quantify the radioactivity incorporated into the P-gp using the STORM 860 PhosphorImager system with a high-sensitivity screen and ImageQuaNT software.

Mass Spectrometry. Protein bands were cut from polyacrylamide gels, and the proteins were in-gel alkylated with 55 mM iodoacetamide for 45 min at 25 °C. Following washing with 50 mM ammonium bicarbonate, the proteins were in-gel trypsin digested for 16 h at 25 °C using a Montage In-Gel Digest Kit (Millipore, Billerica, MA) on a 96-well C18 ZipPlate following the manufacturer's guidelines. Peptides were eluted from the plate, and an aliquot of each digestion reaction was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a MALDI micro MX (Waters, Milford, MA) to look for modified peptides. To determine the sites of modification, the remaining peptides were acidified by 1:1 dilution with Diluent Buffer containing 2% acetonitrile, 0.1% formic acid, and 0.05% HFBA in water. Separation of the peptides was performed at 500 nL/min and was coupled to online analysis by tandem mass spectrometry (nLC-ESI-MS/MS) on an LTQ ion trap mass spectrometer (ThermoElectron, San Jose, CA) equipped with a nanospray ion source. From the diluted peptides, 30 μL was loaded onto a 0.1 × 150 mm Magic C18AQ column (Michrom Bioresources, Inc., Auburn, CA) inline after a nanotrap column using the Paradigm MS4MDLC (Michrom Bioresources, Inc., Auburn, CA). Elution of the peptides into the mass spectrometer was performed with a linear gradient from 95% mobile phase A (2% acetonitrile, 0.5% acetic acid, 97.5% water) to 65% mobile phase B (10% water, 0.5% acetic acid, 89.5% acetonitrile) in 45 min and then to 95% mobile phase B in 5 min. The peptides were detected in positive ion mode using a mass list containing the masses of the modified peptides identified by MALDI-TOF MS analysis.

Molecular Modeling. A Nucleotide Binding Domain homology model of human P-gp was constructed using the X-ray crystal structure of the ABC transporter hemolysin-B (Hly-B), in complex with ATP/Mg^{2+}, as the template (1XEF.pdb).[39] Residues K408, P1045, T1046, R1138, and V1139, missing in the Hyl-B structure, were included in the final model, importing three loops from the X-ray structure of the ABC transporter associated with antigen processing TAP1 (2IXF.pdb).[40] The fragments incorporated into the model were the following: PNHPNVQVLQ (residues 27−36 of TAP1-NBD1); PNHPNVQVLQGL (residues 27−38 of TAP1-NBD2), and LTRTPT (residues 117−122 of TAP1-NBD2). Protein residues were *in silico* mutated to the P-gp sequence, and the side-chain conformations of the new residues were visually inspected and modified when necessary. The structure was energy-minimized by CHARMm.[41] Docking studies of FSBA at the NBDs were carried out using the program Vina-Autodock[42] or by manually positioning the FSBA molecule with its adenine group π-stacked to the Y401 and adjusting the torsion angles of rotatable bonds. After ligand docking, the protein−FSBA structures were energy-minimized by CHARMm.

Fluorescent Substrate Accumulation Assay by Flow Cytometry. Accumulation assays with rhodamine 123 (0.5 μg/mL) were carried out in P-gp-expressing KB-V-1 cells as described previously.[43] For all samples, 10 000 events were counted and analysis was performed with Cell Quest (Becton-Dickinson Immunocytometry systems). The mean fluorescence intensity was calculated using the histogram stat program in Cell Quest software.

RESULTS

FSBA Inhibits Drug Transport in Intact Cells Overexpressing P-gp. FSBA, which is an analogue of ATP, has been used to identify and characterize the nucleotide-binding sites of several ATP-dependent proteins[15] but had not been used in the study of ABC transporters. The structure of FSBA strongly resembles that of ATP, except for the presence of 5′-fluorosulfonylbenzoyl in place of the phosphoryl groups of ATP (Figure 1A,B). We used a flow cytometry-based assay to investigate the effect of FSBA on P-gp-mediated drug transport. Flow cytometry using fluorescent transport substrates of P-gp has been extensively used to measure transport function.[44] Cells overexpressing P-gp accumulate less fluorescent substrate, such as the typically used rhodamine 123 than parental cells not expressing P-gp. Treatment of P-gp-overexpressing cells with modulators that interact with P-gp decreases the efflux of the transport substrate and increases accumulation of the fluorescent substrates to levels seen in the parental cells.[44] Figure 2 clearly shows that KB-V1 cells that overexpress P-gp accumulate lower levels of rhodamine 123 than the parental KB-3-1 cells. Treatment of the KB-V1 cells with the P-gp modulator XR9576 increases rhodamine 123 levels comparable to those observed in KB-3-1 cells. When the P-gp-expressing cells were incubated with 1.5 mM FSBA for 45 min at 37 °C, the accumulation of rhodamine 123 also increased. The increase in the fluorescence signal was equivalent to that observed in the presence of XR9576, which is a well-characterized inhibitor of P-gp-mediated transport.[45] To further elucidate the mechanism of FSBA action, we investigated the effect of FSBA on both P-gp-mediated ATP hydrolysis as well as P-gp drug-substrate interactions.

Characterization of FSBA-Mediated Inhibition of the ATPase Activity of P-gp. As the structure of FSBA strongly resembles that of ATP (Figure 1A,B), we determined its effect on ATP hydrolysis by P-gp. Figure 3A shows that FSBA inhibits P-gp-mediated ATP hydrolysis in a concentration-dependent manner (IC_{50} = 0.21 ± 0.05 mM mean ± SD, n = 3), with the possibility that FSBA has an effect on ATP hydrolysis either by binding directly to the nucleotide-binding domains (NBDs) of P-gp or elsewhere on the molecule. The $t_{1/2}$ for the inhibition of P-gp-mediated ATP hydrolysis by FSBA is 3.75 ± 1.72 min (mean ± SD, n = 3), determined by incubating P-gp with FSBA for increasing lengths of time at 37 °C, prior to initiating ATP hydrolysis (Figure 3B). In addition, we observed that FSBA-mediated inhibition of ATP hydrolysis is temperature-dependent

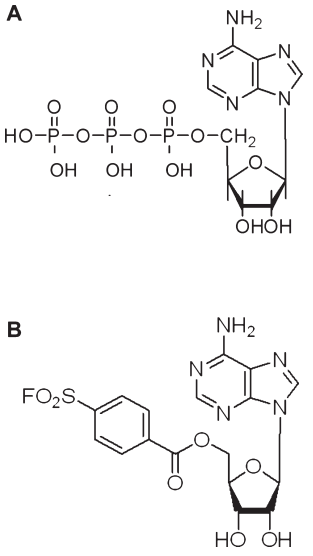

Figure 1. Chemical structures of ATP and FSBA. Comparison of the chemical structures of (A) ATP and (B) FSBA.

(Figure 3C). The time and temperature dependence suggests that inhibition of ATP binding alone is not sufficient for FSBA-mediated inhibition; chemical cross-linking is also necessary. Studies on rabbit pyruvate kinase have shown that the inhibition of this enzyme by FSBA can be partially reversed by the reducing agent dithiothreitol (DTT).[21] The experiments described above were carried out in the absence of DTT. We found that the addition of DTT prevents the FSBA-mediated inhibition of ATPase activity with an apparent EC_{50} of 0.07 mM (Figure S1A). Further experiments were carried out to investigate whether DTT could reverse FSBA-mediated inhibition once the covalent cross-link had formed. Treatment with DTT after incubating P-gp with FSBA for 30 min at 37 °C shows only a modest reversal of this inhibition (Figure S1B: compare III and IV). Thus, if the P-gp is incubated with DTT during FSBA treatment, inhibition of ATPase activity is prevented (Figure S1A); however, once the reaction between FSBA and the amino acid side chains has progressed to completion, it cannot be reversed by DTT (Figure S1B). Thus, the prevention of the FSBA-inhibitory effect of ATP hydrolysis can be ascribed to a quenching effect of DTT on FSBA, when both are added at the same time to initiate the reaction.

[^{14}C]FSBA Cross-Links to Purified P-gp. The work described above suggests that FSBA cross-links to P-gp, and this is established more directly with the data presented in Figure 4. Purified P-gp, reconstituted into proteoliposomes, was incubated with [^{14}C]FSBA for 30 min at 37 °C. The reaction was stopped by the addition of SDS-PAGE loading buffer, and the sample was subjected to gel electrophoresis. In Figure 4A, which is an autoradiogram, we show that the [^{14}C] signal is associated with the P-gp band, demonstrating that the [^{14}C]FSBA is cross-linked to the P-gp. When the [^{14}C]FSBA was incubated with P-gp in the presence of 10 mM ATP, however, there was 50% reduction but not elimination of [^{14}C]FSBA cross-linking to P-gp. In addition, we observed comparable levels of [^{14}C]FSBA cross-linking to wild-type P-gp and a cys-less mutant (Figure 4B), where all seven native cysteine residues have been replaced by alanine. This implies that the cysteine residues are not necessary for the observed cross-linking of FSBA to P-gp.

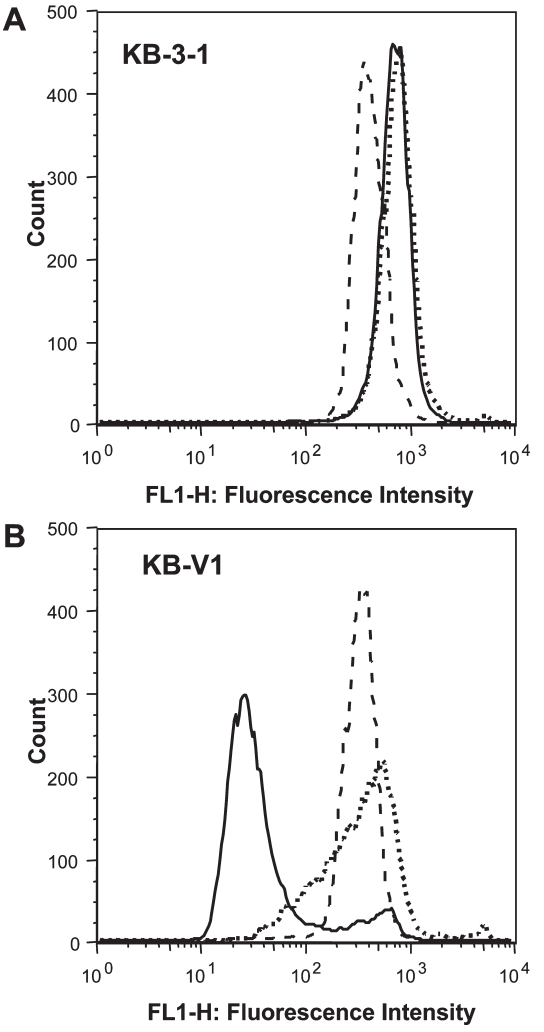

Figure 2. Effect of FSBA on the P-gp-mediated transport of fluorescent substrate. A flow cytometry-based assay showed accumulation of the fluorescent P-gp transport substrate rhodamine 123. Parental, KB-3-1 (A) and P-gp-expressing KB-V1 (B) cells (300 000/tube) were incubated with 0.5 μg/mL of rhodamine 123 for 45 min at 37 °C in the dark, in the absence (control cells: —) or presence of 10 μM XR 9576 (---) or 1.5 mM FSBA (· · ·). The cells were then washed, resuspended in PBS with 0.1% BSA, and analyzed by flow cytometer as described in Materials and Methods. Representative histograms generated with Cell Quest software from three independent experiments are depicted.

FSBA Stimulates ATP Hydrolysis If the Nucleotide-Binding Sites Are Occupied by Saturating Concentrations of ATP. If FSBA binds to the NBDs, it should be possible to test its effect when these sites are occupied by ATP at saturating concentration. In the experiment shown in Figure 5A, the NBDs were occupied with saturating concentration of ATP (5 mM) prior to the addition of FSBA. All additions were made on ice to prevent ATP hydrolysis and cross-linking of FSBA. The ATP hydrolysis was initiated by warming the reaction mixture to 37 °C. Not only was the inhibition of ATPase activity reversed, but there was a >4-fold stimulation of FSBA-mediated ATP hydrolysis; the concentration required for 50% stimulation was 0.07 ± 0.05 mM (Figure 5A). Thus, it is plausible that FSBA also interacts with the substrate-binding sites of P-gp and as a consequence stimulates ATP hydrolysis. Moreover, when the NBDs are occupied by 5 mM ATP, FSBA

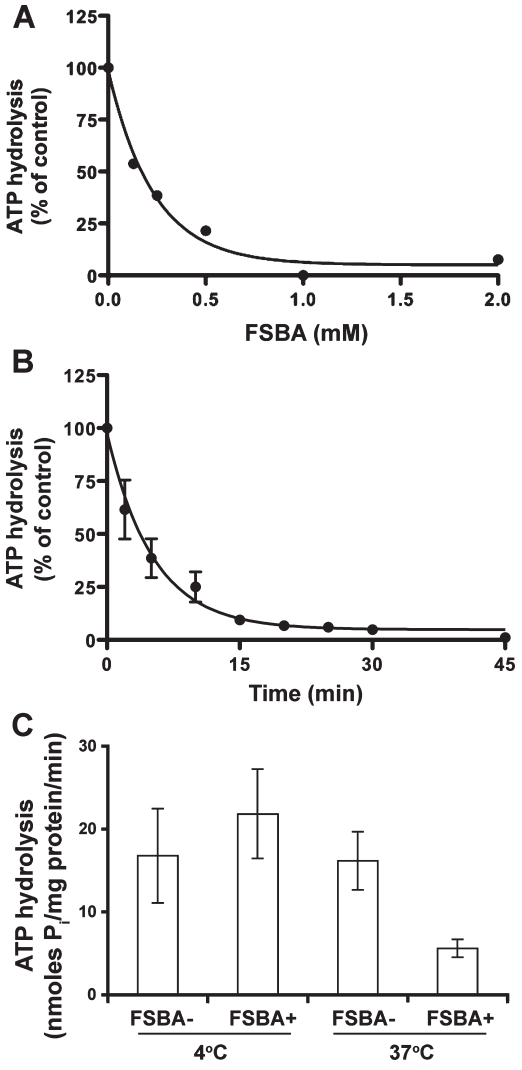

Figure 3. Effect of FSBA on P-gp-mediated ATP hydrolysis. (A) Inhibition of P-gp ATPase activity by FSBA. Vanadate-sensitive P-gp-mediated ATP hydrolysis was measured as described in Materials and Methods in the presence of increasing concentrations of FSBA (0−2 mM). In this experiment the FSBA was incubated with P-gp (10 μg protein/100 μL) for 30 min at 37 °C prior to addition of 5 mM ATP. The IC_{50} (FSBA) for inhibition of P-gp-mediated ATP hydrolysis is 0.21 ± 0.05 mM. A representative plot is shown (n = 3). (B) Time course of the inhibition by FSBA of P-gp-mediated ATP hydrolysis. ATP hydrolysis was measured for 20 min following incubation of P-gp (10 μg/100 μL) with FSBA (1 mM) for increasing amounts of time. The $t_{1/2}$ for the inhibition of ATP hydrolysis was 3.8 min. (C) Effect of temperature on the inhibition of P-gp-mediated ATP hydrolysis by FSBA. P-gp (10 μg/100 μL) was incubated for 30 min in the absence or presence FSBA (1 mM) at either 4 or 37 °C prior to initiating ATP hydrolysis at 37 °C. The reaction conditions are shown on the figure. Data represent the mean values (±SD) obtained from at least three independent experiments.

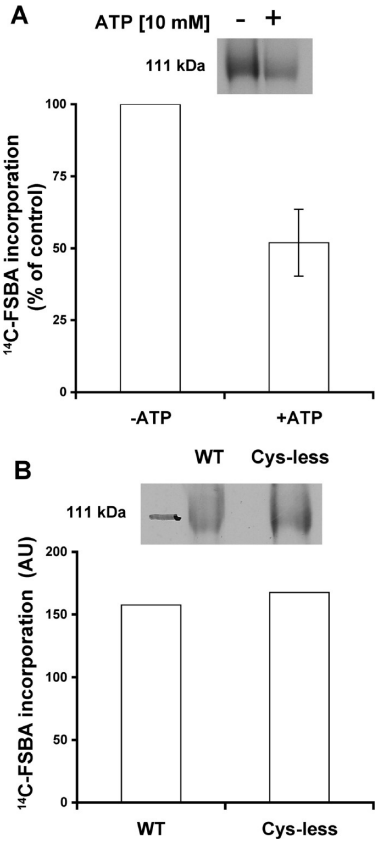

Figure 4. Cross-linking of [^{14}C]FSBA to P-gp. (A) Purified P-gp (10−36 μg protein) reconstituted into proteoliposomes was incubated for 30 min at 37 °C with 0.4 mM [^{14}C]FSBA in the presence or absence of 10 mM ATP. The samples were then electrophoresed, and the dried gel was exposed to an X-ray film at −70 °C for 3−7 days. (B) Purified wild-type P-gp and the cys-less mutant (60 μg protein) was reconstituted into proteoliposomes and incubated for 30 min at 37 °C with 0.35 mM [^{14}C]FSBA. The samples were then electrophoresed, and the dried gel was exposed to an X-ray film at −70 °C for 3−7 days. In both (A) and (B) the section of the autoradiogram with the P-gp band is indicated, and the bar graphs show the mean ± SD values following quantification, which was carried out using the STORM 860 PhosphorImager system with a high-sensitivity screen and the software ImageQuaNT.

affects the V_{max} but not the K_m of P-gp-mediated ATP hydrolysis (Figure 5B), which suggests noncompetitive stimulation of the ATPase reaction (V_{max} 58.9/21.8, K_m 0.64/0.54 mM in the presence/absence of FSBA, respectively). This observation is consistent with the fact that the nucleotide- and substrate-binding sites are located on different domains of P-gp.[9] To permit competition of FSBA and ATP at the NBDs, we incubated FSBA in the presence of ATP for 30 min at 37 °C. This incubation was carried out in the absence of Mg^{2+}, permitting both ATP and FSBA to interact with the NBDs. However, ATP hydrolysis would not occur, as Mg^{2+} is necessary for P-gp-mediated ATP hydrolysis.[46] ATP hydrolysis was initiated by the addition of Mg^{2+} to the reaction mixture. The presence of FSBA results in a ∼3-fold increase in the K_m (ATP) 1.31/0.44 mM in the presence/absence of FSBA, respectively) (Figure 5C). This decrease in the affinity of ATP suggests competitive inhibition (i.e., both FSBA and ATP compete for the same or similar sites on P-gp). As it is not possible to prevent FSBA interaction at the drug-substrate site in these experiments, some of the observed kinetic changes may be influenced by its effect on substrate-binding site in TMDs.

Interaction of FSBA with the Nucleotide- and Substrate-Binding Sites of P-gp. Previous studies have demonstrated that the nucleotide analogue 8-azido[α-^{32}P]ATP photo-cross-links specifically to the NBDs of P-gp and can be displaced by unlabeled nucleotides.[38] We demonstrate in Figure S2 that FSBA inhibits the photo-cross-linking of 8-azido[α-^{32}P]ATP to P-gp in

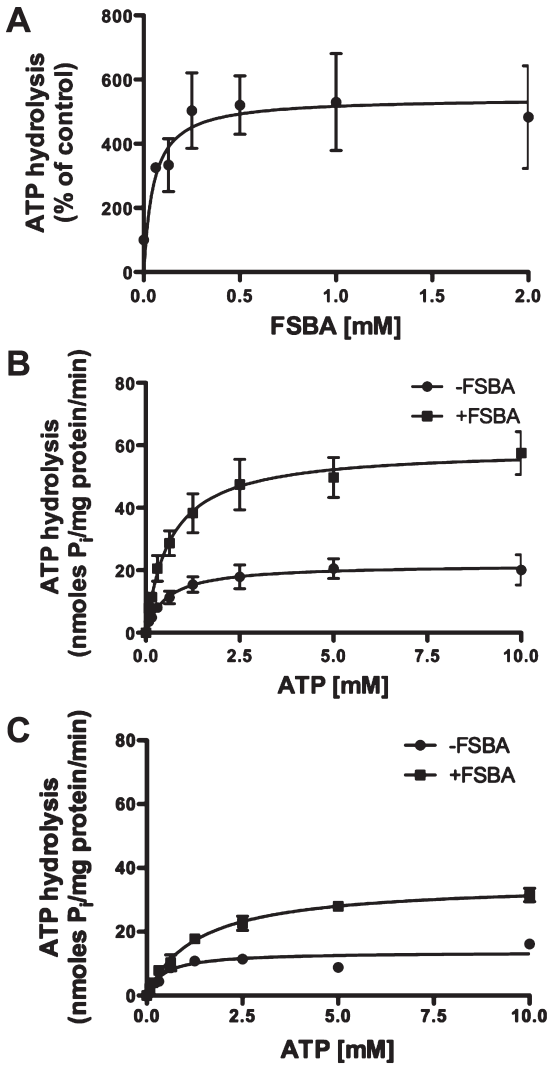

Figure 5. Characterization of the FSBA-mediated stimulation of ATP hydrolysis by P-gp. (A) The experiment was performed as described in Figure 3A, except that a saturating concentration of ATP (5 mM) was added prior to the addition of FSBA. All additions were made on ice, and the ATPase reaction was initiated by warming the tubes to 37 °C. The concentration of FSBA required for 50% stimulation of P-gp-mediated ATPase activity of P-gp is 0.07 ± 0.05 mM. (B) Michaelis−Menten kinetics of P-gp-mediated ATP hydrolysis were determined in the absence (●) or presence (■) of FSBA (2 mM). K_m (ATP) values in the absence and presence of FSBA were 0.54 ± 0.17 and 0.64 ± 0.14 mM, respectively, and the V_{max} values were 21.4 and 58.9 nmol P_i/mg protein/min, respectively. Note that both ATP and FSBA were added at the same time without any prior incubation at 37 °C. (C) Michaelis−Menten kinetics of P-gp-mediated ATP hydrolysis were determined in the absence (●) or presence (■) of FSBA (2 mM). P-gp was incubated in the presence or absence of FSBA (2 mM) and 5 mM ATP for 30 min at 37 °C in the absence of Mg^{2+}. ATP hydrolysis was then initiated by the addition of 10 mM $MgCl_2$. K_m (ATP) values, in the absence and presence of FSBA, were 0.44 ± 0.14 and 1.31 ± 0.18 mM, respectively. The data points in all graphs (A, B, C) represent the mean values ± SD obtained from at least three independent experiments, and the kinetic parameters were estimated using the curve-fitting software GraphPad PRIZM.

a concentration-dependent manner with an IC_{50} of 0.68 ± 0.23 mM (mean ± SD; n = 3). Similarly, the photoaffinity analogue of prazosin, [^{125}I]IAAP, which is transported by P-gp, has been used extensively to study interactions at the substrate-binding sites.[47] Drug substrates of P-gp inhibit the photo-cross-linking of [^{125}I]IAAP, but nucleotides such as ATP and ADP have no effect.[34] Although FSBA is a nucleotide analogue, it inhibits the photo-cross-linking of P-gp with [^{125}I]IAAP with an IC_{50} of 0.17 ± 0.03 mM (Figure 6A). The addition of ATP had no effect on the inhibitory effect of FSBA (IC_{50} = 0.09 ± 0.04 mM; n = 3). Furthermore, similar IC_{50} value (0.20 ± 0.08 mM) was obtained using the double mutant Y401/1044A, in which the tyrosine residues in A-loops of both NBDs are replaced with alanine, and ATP is incapable of binding to the mutant P-gp (Figure 6B).[48] These results suggest that FSBA inhibits photolabeling of P-gp with [^{125}I]IAAP either by directly interacting with substrate-binding sites or by binding elsewhere regardless of its interaction with the NBDs. Moreover, these data indicate that there are two types of interactions between P-gp and FSBA. One is the chemical cross-linking of FSBA to residues within or nearby NBDs affecting ATP sites. The other is an interaction most likely in the TMD region affecting the drug-substrate binding.

When FSBA was incubated with P-gp for 30 min at 4 °C and then washed off by centrifugation, there was no inhibition of ATP hydrolysis (Figure 3C: compare the first two bars). On the other hand, if FSBA was incubated with P-gp at 37 °C prior to removal of free FSBA by centrifugation, there was a significant inhibition of P-gp-mediated ATP hydrolysis (Figure 3C: compare the third and fourth bars). These results suggest that FSBA does indeed cross-link to P-gp, but only at 37 °C and not at 4 °C. On the other hand, P-gp incubated with FSBA at 37 °C and washed by centrifugation had no effect on the cross-linking of IAAP (Figure 6C), suggesting that FSBA fraction that inhibits photolabeling with IAAP is washable, and thus it likely interacts with but does not cross-link to the substrate-binding site(s). Similar results were obtained when the mutant P-gp (Y401A/Y1044A), which lacks the capacity to bind nucleotides, was used in lieu of the wild-type protein (Figure 6D). These data suggest that it is unlikely that cross-linking of FSBA occurs at the drug-substrate-binding sites in the TMDs.

Identification of FSBA-Labeled Sites Using Mass Spectrometry. Purified P-gp in proteoliposomes was cross-linked with 2 mM FSBA by incubation at 37 °C for 30 min in either the presence or absence of 5 mM ATP. These samples were then electrophoresed, and the excised protein bands were subjected to in-gel digestion followed by mass spectrometry analysis, as described in Materials and Methods. Figure 7 shows an example of the MALDI-TOF mass fingerprinting results. The two panels show a portion of the mass spectrum of the P-gp tryptic digest untreated and after treatment with FSBA. As an example, the peptide at m/z = 898.95 is shifted to m/z = 1331.85 following FSBA modification. Table 1 lists all the peptides identified with cross-linking to FSBA. Of the seven peptides identified, four were not cross-linked in the presence of ATP. It is noteworthy that no cross-linked peptides were identified in the TMDs, although peptides from the TMDs were observed in the untreated sample. These data demonstrate that FSBA cross-links to residues within or nearby the NBDs of P-gp, while cross-linking to the TMDs is not detectable.

Molecular Modeling of FSBA Interaction at the NBDs of P-gp. As there is no available X-ray crystal structure of human P-gp, a NBD homology model of P-gp on the basis of the hemolysin transporter (Hly-B) was built as described in Materials and Methods. The Hly-B structure (1XEF.pdb) was selected as a template for the following three reasons: high similarity between sequences at the NBDs, the fact that the Hly-B protein was crystallized with bound ATP and Mg^{2+}, and the acceptable

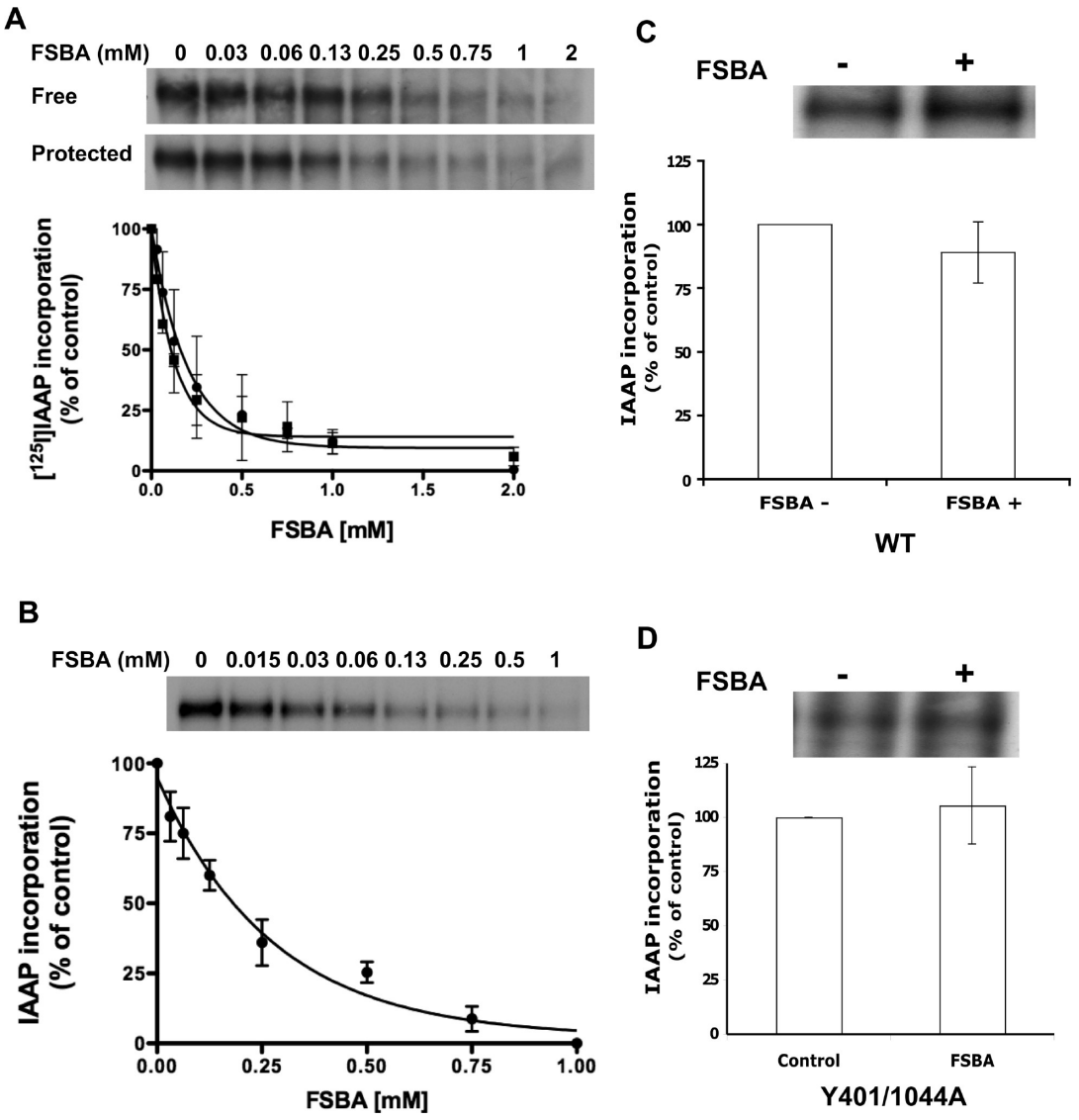

Figure 6. FSBA binds to the drug-substrate site(s) and inhibits photolabeling of P-gp with [^{125}I]IAAP. (A) The apparent affinities of FSBA for the substrate-binding sites of WT-P-gp were estimated by monitoring the cross-linking of the photoaffinity analogue of prazosin, [^{125}I]IAAP in the presence of increasing concentrations of FSBA (0−2 mM) in the absence (●) or presence of 5 mM ATP (■), as described in Materials and Methods. (B) The apparent affinity of FSBA for the substrate-binding sites of the Y401A/Y1044A mutant-P-gp, which cannot bind ATP, was estimated as described in (A). For experiments in (A) and (B), 5−7 nM [^{125}I]IAAP was used. Photoaffinity labeling of wild-type (C) and Y401A/1044A mutant (D) with IAAP after the formation of FSBA-P-gp adduct. P-gp containing crude membranes (500 μg protein) was treated with or without FSBA (2 mM) in 1 mL of ATPase buffer for 30 min at 37 °C. The samples were washed by centrifugation at 200000*g* for 20 min at 4 °C to remove the excess FSBA, resuspended in ice-cold 150 μL of ATPase buffer, and 10 μL of washed membranes (50−75 μg of protein) were incubated with 10 nM IAAP and photo-cross-linked as described in Materials and Methods. Finally, all samples were electrophoresed and the dried gel was exposed to an X-ray film. The upper panel depicts the autoradiogram from a representative experiment, and similar results were obtained in two additional experiments. The radioactivity associated with the P-gp band was quantified and represented in the lower panels using a phosphorimager and the curve-fitting software GraphPad PRIZM (for panels A and B). In (A) upper, an autoradiogram depicts FSBA treatment in the absence (free) and the presence of 5 mM ATP (protected).

resolution of the structure (2.5 Å). The molecular modeling studies were carried out with the aim of better understanding the biochemical data presented here: (i) FSBA and ATP compete for the same site at the NBDs of P-gp and (ii) chemical cross-linking of FSBA to residues within or nearby NBDs. The highly similar structures of FSBA and ATP suggest that FSBA binds at the ATP binding site in the same fashion as ATP, with the adenine group π-stacked to the A-loop tyrosine 401 of NBD1 or the Y1044 of NBD2.[48,49] This typical binding mode set the conditions for the modeling studies. The program Vina-autodock was used to predict the possible noncovalent binding of FSBA at the nucleotide-binding sites. In fact, FSBA fits effectively at the NBDs with the adenine group interacting with Y401 (NBD1) or Y1044 (NBD2) through $\pi-\pi$ contact and the fluorosulfonyl moiety at the approximate position typically occupied by the ATP β- and γ-phosphate (see Figure 8A,B). However, this pocket is surrounded by positively charged residues such as Walker A lysine-433 and also a Mg^{2+} ion, which creates an environment that is better suited for negatively charged ligands (ATP) than neutral ones (FSBA).

Table 1. FSBA-Cross-Linked Peptides in P-gp[a]

peptide	m/z	P-gp residue numbers	sequence	NBD	ATP prevents cross-linking[b]	sarget residue	comments
1	715.99	790−794	YMVFR	—[c]	no		distal to NDB1
2	898.95	409−416	ILKGLNLK	1	no	Lys-411	within the NBD1 region
3	944.92	610−617	GNHDELMK	1	yes		distal to the H-loop in NBD1
4	1015.73	1094−1102	VLLDGKEIK	2	yes	Lys-1102	within the NBD2
5	1107.45	396−404	NVHFSYPSR	1	no	His-398[e]	very close to the A-loop (Y401) in NBD1
6	1910.55	443−459	LYDPTEGMVSVDGQDIR	1	yes	Arg-459[e]	within the NBD1
7	2249.76	625−645	LVTMQTAGNEVELENAADESK	—[d]	yes		distal to NBD1 in the linker region

[a] The FSBA labeled purified P-gp bands excised from NuPage gels were subjected to in-gel trypsin digestion followed by MALDI-TOF MS (see legend to Figure 7 for experimental conditions). [b] Purified P-gp incubated with 5 mM ATP before addition of FSBA. "No" indicates cross-linking with FSBA is not prevented by pretreatment with ATP, whereas "yes" indicates inhibition by ATP. [c] Intracellular loop 3 (ICL3). [d] Linker region, most of the peptide outside the NBD1 region. [e] Potential target residues.

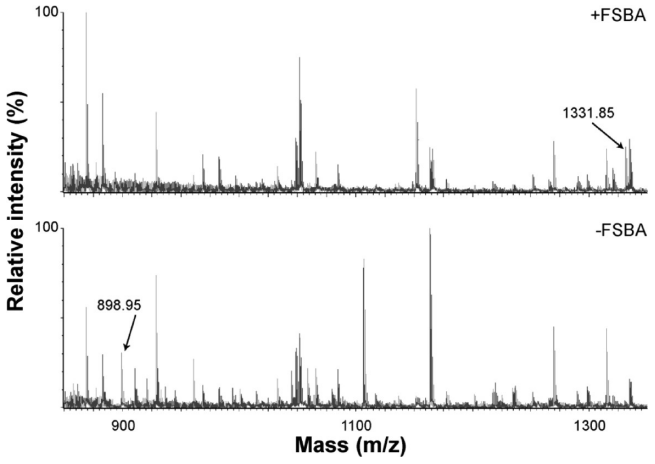

Figure 7. Identification of FSBA-labeled sites using MALDI-TOF MS. Purified and reconstituted P-gp (10−20 μg protein) was incubated with 2 mM FSBA at 37 °C for 30 min in either the presence or absence of 5 mM ATP. The samples were run on a 7% NuPage gel at constant voltage (150 V). Protein (P-gp) bands excised from polyacrylamide gels were subjected to in-gel trypsin digestion followed by MALDI-TOF MS, as described in the Materials and Methods. Representative regions of the MS spectrum of control, i.e., untreated (bottom), or FSBA-treated (top) are shown. The peak at m/z = 898.95 represents an unmodified peptide (peptide 2 in Table 1) that shifts to m/z = 1331.85 following FSBA modification (marked with an arrow).

Further modeling was carried out to investigate other FSBA−protein interactions that better account for the efficient inhibition of ATP hydrolysis. The electrophilic fluorosulfonyl group of FSBA is reactive toward Lewis bases such as the side chains of lysine, tyrosine, histidines, serine, and cysteine[15] and responsible for the chemical cross-linking to the NBDs of P-gp. Therefore, modeling studies having both (i) the FSBA adenine moiety π-stacked to the Y401 and (ii) the fluorosulfonyl group pointed to any basic residue of the fragments identified as cross-linked to FSBA by LC/MS/MS (see Table 1) were performed. One FSBA molecule was successfully modeled with the fluorosulfonyl group pointing toward the amino group of Lys-411 (Figure S3), thus offering an explanation for the origin of peptide fragment 2 detected by LC/MS/MS (see Figures 7 and 8C,D and Table 1) and also providing an alternative mode that accounts for the efficient inhibition of ATP hydrolysis by FSBA. Interestingly, when FSBA is modeled at NBD2, the fluorosulfonyl group points to a glutamine residue (Q1054, the equivalent residue of K411 at NBD2), a much weaker Lewis base than lysine, providing a plausible explanation as to why the equivalent fragment of NBD1 at NBD2 is not found cross-linked to FSBA. Although FSBA could not be docked to explain the origin of the rest of the peptide fragments detected by MS, it is important to mention that just a small movement of loops next to the ATP site would make possible docking with the adenine moiety π-stacked to the A-loop tyrosine and the reactive $-SO_2F$ group pointing to K1102 (fragment 4 in Table 1), to H398 (fragment 5), and R459 (fragment 6). Although arginine has not been reported frequently as a target for FSBA, it has also been found cross-linked to FSBA.[50] The location of FSBA peptides labeled 2−7 (Table 1) in the homology model of human P-gp NBDs is shown in Figure 8C,D. Earlier reports indicated that FSBA could react with several different nucleophilic amino acids.[17,20] While lysine, tyrosine, and histidine residues are part of the sequence of the FSBA-cross-linked peptides, no cysteine is found in any of them. Our results with the Cys-less P-gp (see Figure 4B) and FSBA-labeled P-gp peptides (Table 1) are also in agreement with these studies.

A homology model of human P-gp on the basis of the recently published X-ray structure of mouse P-gp was also built and subjected to docking analysis. The mouse P-gp sequence is very similar to human P-gp (87% identity), but the X-ray structure reported has two significant disadvantages: low resolution (3.8 Å) and the fact that it was obtained in the absence of nucleotide. Interestingly, we found that FSBA (as well as ATP) cannot be docked in the same way as in the model based on Hly-B structure because the conformation of the Walker A motif is different (Figure S4). This suggests that a homology model of NBDs of human P-gp based on Hly-B structure (with bound ATP/Mg^{2+}) is the appropriate one for docking studies of FSBA.

DISCUSSION

In this report, we have characterized in considerable detail the interactions of FSBA with P-gp. The main reason for this is that FSBA is an ATP analogue, and it has been used extensively to chemically modify and probe the nucleotide-binding sites of proteins that bind and hydrolyze nucleotides.[14] This could thus also be a useful reagent to study the NBDs of P-gp as well as other ABC transporters.

The reports in the literature primarily consider FSBA as a nucleotide analogue[14] for kinases and other ATP-binding proteins. However, our results show that FSBA interacts with NBDs and also has an effect on the substrate-binding sites of P-gp

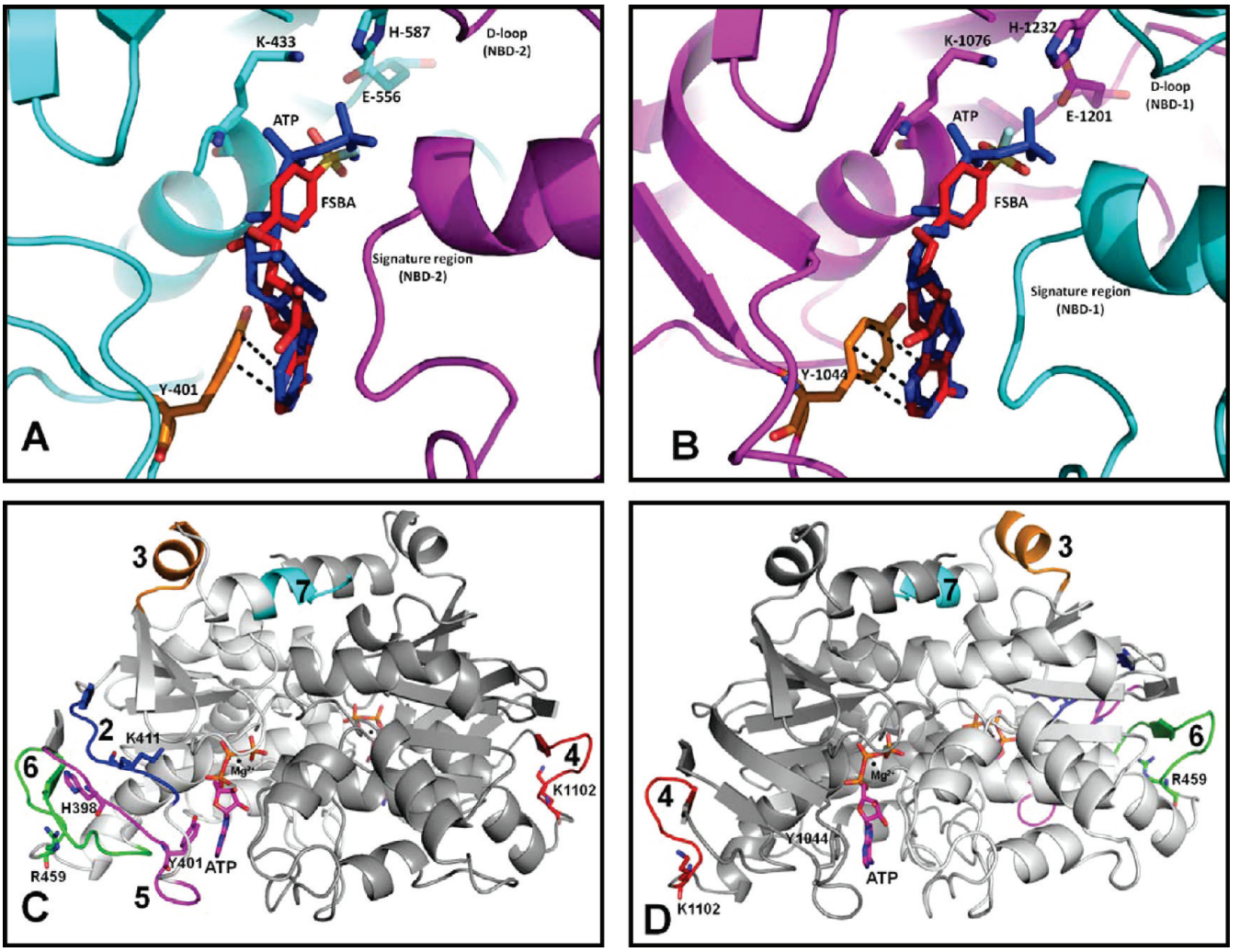

Figure 8. Modeling of FSBA in NDBs of human P-gp. The homology model of NBDs of human P-gp using the HlyB NBD structure was generated as described in Materials and Methods. (A) Modeling of FSBA at the NBD1. The FSBA molecule is docked at the NBD1 with its adenine group π-stacked to the aromatic ring of Y-401 (A-loop). An original FSBA conformation was generated by the program Vina-Autodock, and then the protein−FSBA complex was energy-minimized using CHARMm. (B) Modeling of FSBA at the NBD2. The FSBA molecule is docked at nucleotide-binding site 2 with its adenine group π-stacked to the aromatic ring of Y-1044 (A-loop). An original FSBA conformation was built manually into the NBD2 through molecular torsions, and then the protein−FSBA complex was energy-minimized using CHARMm. (C, D) Location of the FSBA-labeled peptides in the model of human P-gp NBDs. The peptides are painted in different colors and numbered the same as in Table 1. Target residues are also shown in stick models and labeled. The NBD model is shown in two orientations: (C) NBD1 to the left and NBD2 to the right and (D) NBD2 to the left and NBD1 to the right. Parts A and B are colored as follows: NBD1 = cyan, NBD2 = magenta; ATP = blue and FSBA = red; Y-401/Y-1044 = orange. Parts C and D: NBD1 = light gray, NBD2 = dark gray; O = red, N = blue, S = yellow, F = light blue, P = orange, Mg^{2+} = black ball (only shown in C and D); and created with PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC).

(Figures 2, 5A, and 6). In intact cells, FSBA, similar to other P-gp inhibitors (XR9576), blocked the efflux of rhodamine 123 (Figure 2), suggesting that FSBA inhibits P-gp mediated transport in intact cells by interacting at the transport-substrate sites in the TMDs rather than in the NBDs, as one would expect that the NBD sites are occupied by ATP under physiological conditions with the intracellular concentration of ATP in the range of 3−5 mM. Most nucleotides (and nucleotide analogues) are not transport substrates of P-gp, and the transport substrates do not directly interact with the NBDs.[10] This is consistent with a large body of work that shows that ATP hydrolysis and drug-substrate transport occur at different domains.[9] The TMDs are anchored in the plasma membrane and interact with the transport substrates which are generally hydrophobic, while the NBDs, which are physically located in the cytoplasm, interact with hydrophilic nucleotides.[9] In FSBA, the benzoylsulfonyl fluoride replaces the triphosphate moiety of ATP (Figure 1B). As a result, unlike ATP, which is negatively charged, FSBA is neutral[15] and there is the addition of an aromatic benzoyl group. These changes make FSBA more hydrophobic (log P = 2.64), thus penetrating the membrane domains and providing it with structural features that characterize transport substrates of P-gp. This can explain how FSBA is able to interact with the drug-substrate-binding sites of P-gp. We provide evidence for the effect of FSBA on the substrate-binding sites in Figure 6A,B. The prazosin analogue IAAP has been used extensively as a photoaffinity probe for the substrate-binding sites of P-gp.[47] The concentration-dependent inhibition of IAAP binding to P-gp

by FSBA (Figure 6A,B) demonstrates that FSBA interacts directly with the substrate-binding site(s). It has been pointed out previously that the replacement of the phosphate groups of ATP with the sulfonyl fluoride group makes FSBA uncharged and a less desirable ATP analogue;[15] however, these modifications make FSBA more accessible to the substrate-binding site(s) (see above). In addition, there appear to be different energetic requirements for binding to the ATP sites and chemical cross-linking. The former can be demonstrated by the virtually instantaneous displacement of 8-azido[α-^{32}P]ATP by FSBA at 4 °C. On the other hand, the irreversible FSBA-mediated inhibition of ATP hydrolysis (as a consequence of chemical cross-linking) has a $t_{1/2}$ of ~4 min (Figure 3B), and this inhibition is strongly temperature-dependent (Figure 3C). It is clear that while the reversible binding of FSBA to NBDs is temperature-independent, the chemical cross-linking to residues within and nearby NBDs is temperature-dependent.

The sulfonyl fluoride group of FSBA, which replaces the phosphate groups of ATP, is a reactive functional group that can react with several amino acids.[16−18] It has been argued that as the sulfonyl fluoride participates in a broad range of reactions, there is a reasonable probability of reaction within any particular active site.[15] To distinguish between covalent and noncovalent interactions, P-gp was incubated for 30 min in the absence or presence of FSBA. Reactions were carried out either at 4 or 37 °C, and the excess unreacted FSBA was removed by dilution with cold buffer followed by centrifugation. The P-gp samples that were incubated with FSBA at 37 °C showed a strong inhibition of ATP hydrolysis (Figure 3C: compare third and fourth bars), while those incubated at 4 °C showed no inhibition (Figure 3C: compare first and second bars). The control (incubation at 4 °C) shows that unreacted FSBA can be effectively removed by centrifugation, and it is only the chemically cross-linked FSBA that inhibits ATP hydrolysis. The binding of the transport substrate IAAP to wild-type P-gp or mutant Y401A/1044A (that cannot bind to ATP) is not affected by the formation of an FSBA-P-gp adduct (Figure 6C,D), suggesting that FSBA is not cross-linked to the IAAP binding site. Thus, the interaction of FSBA at the drug-substrate binding pocket in TMDs is reversible, and it does not depend on the functional status of ATP sites (Figure 6A,B).

The covalent cross-linking of FSBA to P-gp is further verified by cross-linking of [^{14}C]-labeled FSBA to purified P-gp reconstituted into proteoliposomes (Figure 4A). In addition, we used mass spectrometry to identify sites on P-gp labeled with FSBA (Figure 7). Seven peptides were labeled with FSBA, of which four were protected by pretreatment with 5 mM ATP (Table 1). This is consistent with the observation that incubation with a saturating concentration of ATP prior to addition of FSBA provides only partial protection to labeling by [^{14}C]FSBA (Figure 4A). Identification of the peptides labeled with FSBA further suggests that FSBA cross-links mainly to residues within or near the NBDs (five out of seven fragments are within or near the ATP sites; Table 1 and Figures 7 and 8C,D), despite interrogating both the nucleotide- and substrate-binding sites (Figures 3 and 6).

Previous studies have shown that FSBA participates in covalent reactions with various amino acids (for review see ref 15), including tyrosine, lysine, histidine, serine, and cysteine. The X-ray crystal structures of the NBD domains of several ABC transporters (e.g., MJ0796-1L2T.pdb; Hly-B-1XEF.pdb) show that the ATP molecule is sandwiched between the A-loop, the Walker A and B domains, and the H-loop of one NBD and the signature sequence and D-loop of the opposing NBD.[39,51] Docking studies of FSBA at both ATP sites show the sulfonyl fluoride moiety of FSBA (which is the reactive group) is located close to the position normally occupied by the β- and γ-phosphate of ATP (see Figure 8A,B). Therefore, residues such as the cysteine, serine, and lysine of Walker A, the histidine of the H-loop, and the serine of the signature motif are potential targets for FSBA. However, all those residues (except the H-loop histidine) are not properly oriented for chemical reaction to the partially positively charged sulfur of FSBA. The H-loop histidine seems to be suitably oriented for reaction, but is too far (more than 5 Å) from the reactive fluorosulfonyl group. In addition, LC/MS/MS analysis of P-gp labeled with FSBA did not yield any fragment with residues responsible for binding ATP (Figure 7 and Table 1). On the other hand, modeling studies yielded a reasonable explanation for the origin of fragment 2, and further structural analysis revealed that just small conformational changes at loops close to the ATP sites are necessary to explain the origin of fragments 4−6. However, the fact that ATP does not prevent cross-linking of three fragments out of seven suggests that FSBA is also binding to P-gp at other sites near the NBDs (see comments column in Table 1).

Tyrosine kinase inhibitors, which are developed based on their ability to bind to the catalytic site and prevent ATP binding (gleevec, nilotinib, etc.), interact at the drug-substrate sites of P-gp and ABCG2 but not at the ATP sites.[52] FSBA is, to our knowledge, the first ATP analogue that has an effect on the both the drug-substrate and ATP sites of P-gp, providing a unique and useful tool for biochemical studies of other ABC drug transporters including ABCG2, ABCC1, ABCC2, ABCC4, and ABCC7.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Effect of DTT on FSBA-P-gp interactions (Figure S1), the inhibition of photolabeling of P-gp with 8-azido [α-^{32}P]ATP by FSBA (Figure S2), modeling of cross-linking of FSBA to peptide 2 in NBD1 (Figure S3), and alignment of homology models of NBDs of human P-gp based on HlyB and mouse P-gp structures and docking of FSBA (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

ABC, ATP binding cassette; FSBA, 5′-fluorosulfonylbenzonyl 5′-adenosine; IAAP, iodoarylazidoprazosin; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MDR, multidrug resistance; NBD, nucleotide binding domain; P-gp, P-glycoprotein; TMD, transmembrane domain; Vi, sodium orthovanadate.

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