Molecular Pharmacology

Supplemental Material

for

Evidence for the Interaction of A_3 Adenosine Receptor Agonists at the Drug Binding Site(s) of Human P-glycoprotein (ABCB1)

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Supplemental Methods

Chemical synthesis

Materials and instrumentation

All reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). ^{1}H NMR spectra were obtained with a Bruker 400 spectrometer using $CDCl_3$, CD_3OD and DMSO as solvents. Chemical shifts are expressed in δ values (ppm) with tetramethylsilane (δ 0.00) for $CDCl_3$ and water (δ 3.30) for CD_3OD. NMR spectra were collected with a Bruker AV spectrometer equipped with a z-gradient [^{1}H, ^{13}C, ^{15}N]-cryoprobe. TLC analysis was carried out on glass sheets precoated with silica gel F254 (0.2 mm) from Aldrich. The purity of final nucleoside derivatives was checked using a Hewlett−Packard 1100 HPLC equipped with a Zorbax SB-Aq 5 μm analytical column (50 × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). Mobile phase: linear gradient solvent system, 5 mM TBAP (tetrabutylammoniumdihydrogenphosphate)−CH_3CN from 80:20 to 0:100 in 13 min; the flow rate was 0.5 mL/min. Peaks were detected by UV absorption with a diode array detector at 230, 254, and 280 nm. All derivatives tested for biological activity showed >95% purity by HPLC analysis (detection at 254 nm). Low-resolution mass spectrometry was performed with a JEOL SX102 spectrometer with 6-kV Xe atoms following desorption from a glycerol matrix or on an Agilent LC/MS 1100 MSD, with a Waters (Milford, MA) Atlantis C18 column. High resolution mass spectroscopic (HRMS) measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters) using external calibration with polyalanine, unless noted. Observed mass accuracies are those expected based on known performance of the instrument as well as trends in masses of standard compounds observed at intervals during the series of measurements. Reported masses

are observed masses uncorrected for this time-dependent drift in mass accuracy. All of the reagents were purchased from Sigma-Aldrich (St. Louis, MO) and Enamine (Cincinnati, OH).

Ethyl (3*aR*,3*bS*,4*aS*,5*R*,5*aS*)-5-(6-((3-azidobenzyl)amino)-2-iodo-9*H*-purin-9-yl)-2,2-dimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-*d*][1,3]dioxole-3b(3*aH*)-carboxylate (26)

3-Azido-benzylamine (60.1 mg, 0.40 mmol) and Et_3N (0.11 mL, 0.81 mmol) was added to a solution of compound **25** (41 mg, 0.08 mmol) in methanol (2 mL), and it was stirred at room temperature overnight. Solvent was evaporated, and the residue was purified on flash silica gel column chromatography (hexane: ethyl acetate = 1:1) to give the compound **26** (39 mg, 78%) as a colorless foam. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.95 (s, 1H), 7.34 (t, *J* = 8.0 Hz, 1H), 7.22 (d, *J*= 8.0 Hz, 1H), 7.14 (s, 1H), 6.97-6.95 (m, 1H), 5.82 (d, *J*= 7.2 Hz, 1H), 4.94 (s, 1H), 4.81 (d, *J*= 6.4 Hz, 1H), 4.71 (br s, 2H), 4.33-4.26 (m, 2H), 2.26-2.22 (m, 1H), 1.64-1.60 (m, 1H), 1.53-1.49 (m, 4H), 1.34 (d, *J*= 7.2 Hz, 3H), 1.28 (s, 3H). HRMS calculated for $C_{24}H_{26}N_8O_4I$ $(M + H)^+$: 617.1122; found 617.1132.

(3*aR*,3*bS*,4*aS*,5*R*,5*aS*)-5-(6-((3-azidobenzyl)amino)-2-iodo-9*H*-purin-9-yl)-*N*,2,2-trimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-*d*][1,3]dioxole-3b(3*aH*)-carboxamide (27)

40% $MeNH_2$ solution (3 mL) was added to a solution of compound **26** (39 mg, 0.6 mmol) in methanol (3 mL), and it was stirred at room temperature overnight. Solvent was evaporated, and the residue was purified on flash silica gel column chromatography (CH_2Cl_2: MeOH = 30:1) to give the compound **27** (30 mg, 79%) as a colorless syrup. ^{1}H

NMR (CD_3OD, 400 MHz) δ 7.97 (s, 1H), 7.34 (t, *J* = 8.0 Hz, 1H), 7.22 (d, *J*= 7.6 Hz, 1H), 7.14 (s, 1H), 6.97 (d, *J*= 8.0 Hz, 1H), 5.71 (d, *J*= 6.4 Hz, 1H), 4.93 (s, 1H), 4.85 (d, *J*= 7.2 Hz, 1H), 4.71 (br s, 2H), 2.90 (s, 3H), 2.15-2.11 (m, 1H), 1.54-1.48 (m, 4H), 1.39 (t, *J*= 5.2 Hz, 1H), 1.30 (s, 3H). HRMS calculated for $C_{23}H_{25}N_9O_3I$ $(M + H)^+$: 602.1125; found 602.1130.

(3*aR*,3*bS*,4*aS*,5*R*,5*aS*)-5-(6-((3-(4-(3,4-difluorophenyl)-1*H*-1,2,3-triazol-1-yl)benzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9*H*-purin-9-yl)-*N*,2,2-trimethyltetrahydrocyclopropa[3,4] cyclopenta[1,2-*d*][1,3]dioxole-3b(3a*H*)-carboxamide (28)

$PdCl_2(PPh_3)_2$ (7.0 mg, 0.009 mmol), CuI (1.0 mg, 0.004 mmol), 3,4-difluoro-phenylethynyl (36 µL, 0.29 mmol) and triethylamine (69.0 µL, 0.49 mmol) were added to a solution of compound **27** (30 mg, 0.04 mmol) in anhydrous DMF (1 mL), and the solution was heated at 65 ^oC for 2 h. Solvent was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (CH_2Cl_2: MeOH = 35:1) to give the compound **28** (25 mg, 67%) as a colorless syrup. ^1H NMR (CD_3OD, 400 MHz) δ 8.89 (s, 1H), 8.21 (s, 1H), 8.04 (s, 1H), 7.77-7.72 (m, 2H), 7.67-7.62 (m, 1H), 7.61-7.55 (m, 3H), 7.46-7.42 (m, 1H), 7.35-7.28 (m, 2H), 5.79 (d, *J*= 5.2 Hz, 1H), 5.00 (s, 1H), 4.84 (d, *J*= 6.4 Hz, 1H), 2.73 (s, 3H), 2.16-2.12 (m, 1H), 1.53-1.49 (m, 4H), 1.41 (t, *J*= 4.4 Hz, 1H), 1.28 (s, 3H). HRMS calculated for $C_{39}H_{32}N_9O_3F_4$ $(M + H)^+$: 750.2564; found 750.2570.

(1*S*,2*R*,3*S*,4*R*,5*S*)-4-(6-((3-(4-(3,4-difluorophenyl)-1*H*-1,2,3-triazol-1-yl)benzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9*H*-purin-9-yl)-2,3-dihydroxy-*N*-methylbicyclo[3.1.0]hexane-1-carboxamide (8)

A solution of compound **28** (25 mg, 0.03 mmol) in methanol (2.5 mL) and 10% trifluromethane sulfonic acid (2.5 mL) was heated at 70 ^{o}C for 3 h. Solvent was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (CH_2Cl_2:MeOH = 25:1) to give the compound **8** (19.6 mg, 83%) as colorless syrup. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.85 (s, 1H), 8.13 (s, 1H), 8.02 (s, 1H), 7.77-7.74 (m, 2H), 7.66-7.63 (m, 1H), 7.55-7.51 (m, 2H), 7.49-7.46 (m, 1H), 7.40-7.34 (m, 3H), 4.01 (d, *J* = 6.4 Hz, 1H), 2.83 (s, 3H), 2.12-2.08 (m, 1H), 1.87 (t, *J* = 4.8 Hz, 1H), 1.40-1.36 (m, 1H). HRMS calculated for $C_{36}H_{28}N_9O_3F_4$ (M + H) $^+$: 710.2251; found 710.2261.

(3*aR*,3*bS*,4*aS*,5*R*,5*aS*)-5-(6-((3-azidobenzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9*H*-purin-9-yl)-*N*,2,2-trimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-*d*][1,3]dioxole-3b(3a*H*)-carboxamide (29)

$PdCl_2(PPh_3)_2$ (4.43 mg, 0.006 mmol), 3,4-difluoro-phenylethynyl (12 µL, 0.09 mmol) and triethylamine (44.0 µL, 0.31 mmol) were added to a solution of compound **27** (19 mg, 0.03 mmol) in anhydrous DMF (1 mL), and it was stirred at room temperature overnight. Solvent was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (CH_2Cl_2: MeOH = 30:1) to give the compound **29** (17 mg, 88%) as a colorless syrup. ^{1}H NMR (CD_3OD, 400 MHz) □ 8.15 (s, 1H), 7.69-7.64 (m, 1H), 7.55-7.52 (m, 1H), 7.40-7.33 (m, 2H), 7.21 (d, *J* = 8.0 Hz, 1H), 7.16 (s, 1H), 6.98 (d, *J* = 8.0 Hz, 1H), 5.80 (d, *J* = 7.2 Hz, 1H), 5.03 (s, 1H), 4.86-4.84 (m, 3H), 2.77 (s, 3H), 2.18-2.15

(m, 1H), 1.56-1.53 (m, 4H), 1.43 (t, *J* = 5.2 Hz, 1H), 1.31 (s, 3H). HRMS calculated for $C_{31}H_{28}N_9O_3F_2$ $(M + H)^+$: 612.2283; found 612.2283.

(1*S*,2*R*,3*S*,4*R*,5*S*)-4-(6-((3-azidobenzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9*H*-purin-9-yl)-2,3-dihydroxy-*N*-methylbicyclo[3.1.0]hexane-1-carboxamide (7)

Compound **7** (87%) was prepared from compound **29** following the same method as for compound **8**.^{1}H NMR (CD_3OD, 400 MHz) δ 8.14 (s, 1H), 7.60-7.55 (m, 1H), 7.49-7.45 (m, 1H), 7.39-7.32 (m, 2H), 7.21 (d, *J* = 8.0 Hz, 1H), 7.16 (s, 1H), 6.98-6.95 (m, 1H), 5.05 (d, *J* = 6.8 Hz, 1H), 4.89 (s, 1H), 4.86-4.82 (m, 2H), 4.03 (d, *J* = 6.4 Hz, 1H), 2.84 (s, 3H), 2.13-2.10 (m, 1H), 1.88 (t, *J* = 5.2 Hz, 1H), 1.41-1.37 (m, 1H). HRMS calculated for $C_{28}H_{24}N_9O_3F_2$ $(M + H)^+$: 572.1970; found 572.1970

***N*'-(3-(5,5-difluoro-7,9-dimethyl-5*H*-4λ^4,5λ^4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-yl)propanoyl)-3-ethynylbenzohydrazide (31)**

3-Ethyn-benzoic acid (3 mg, 0.02 mmo), HATU (8 mg, 0.02 mmol) and DIPEA (3.7 µL, 0.2 mmol) were added to a solution of compound **30** (5 mg, 0.01 mmol) in dry DMF (0.6 mL), and it was stirred at room temperature overnight. Solvent was evaporated, and the residue was purified on flash silica gel column chromatography (hexane: ethyl acetate = 1:2) to give the compound **31** (5 mg, 71%) as a yellow syrup. ^{1}H NMR (CD_3OD, 400 MHz) □ 8.00 (s, 1H), 7.90 (d, *J* = 8.0 Hz, 1H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.49 (t, *J* = 8.0 Hz, 1H), 7.45 (s, 1H), 7.04 (d, *J* = 3.6 Hz, 1H), 6.45 (d, *J* = 4.0 Hz, 1H), 6.23 (s, 1H), 3.62 (s, 1H), 2.77 (t, *J* = 7.6 Hz, 2H), 2.53 (s, 3H), 2.30 (s, 3H), 1.32-1.30 (m, 2H). HRMS calculated for $C_{23}H_{21}N_4O_2F_2BNa$ (M + Na): 457.1623; found 457.1615

(1*S*,2*R*,3*S*,4*R*,5*S*)-4-(6-((3-(4-(3-(2-(3-(5,5-difluoro-7,9-dimethyl-5*H*-4λ^4,5λ^4-dipyrrolo[1,2-c:2',1'-*f*][1,3,2]diazaborinin-3-yl)propanoyl)hydrazine-1-carbonyl)phenyl)-1*H*-1,2,3-triazol-1-yl)benzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9*H*-purin-9-yl)-2,3-dihydroxy-*N*-methylbicyclo[3.1.0]hexane-1-carboxamide (24)

Compound **31** (5 mg, 0.011 mmol) was added to a solution of compound **7** (4.76 mg, 0.008 mmol) in a mixture of DMF (1.0 mL) and water (1.0 mL). Subsequently freshly prepared 1M sodium ascorbate solution (8.3 µL, 0.008 mmol) followed by 7.5% solution of copper sulphate (13.8 µL, 0.004 mmol) was added into the reaction mixture, and it was stirred at room temperature overnight. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH_2Cl_2:MeOH = 15:1) to give the compound **24** (7.4 mg, 89%) as an orange syrup. ^{1}H NMR (CD_3OD, 400 MHz) δ 8.95 (s, 1H), 8.40 (s, 1H), 8.15-8.12 (m, 2H), 8.07 (s, 1H), 7.92-7.82 (m, 3H), 7.58-7.54 (m, 1H), 7.45-7.43 (m, 2H), 7.37-7.29 (m, 2H), 7.04 (d, *J* = 3.6 Hz, 1H), 7.04 (d, *J* = 3.6 Hz, 1H), 6.46 (d, *J* = 4.0 Hz, 1H), 6.23 (s, 1H), 5.07 (d, *J* = 6.0 Hz, 1H), 4.99 (br s, 2H), 4.89 (s, 1H), 4.07 (d, *J* = 6.4 Hz, 1H), 2.83-2.78 (m, 5H), 2.53 (s, 3H), 2.30 (s, 3H), 2.13-2.09 (m, 1H), 1.87 (t, *J* = 5.2 Hz, 1H), 1.40-1.30 (m, 3H).). HRMS calculated for $C_{51}H_{45}BN_{13}O_5F_4$ $(M + H)^+$: 1006.3696; found 1006.3680

Supplemental Table 1

Taxol (Paclitaxel)		Compound 3	
#	Residue	Residue	Number of Poses
0	L65	L65	1
1	M68	NI	NI
2	M69	NI	NI
3	W232	NI	NI
4	F303	F303	18
5	I306	I306	15
6	Y307	Y307	18
7	Y310	Y310	15
8	F336	F336	12
9	L339	L339	12
10	I340	I340	12
11	F343	F343	16
12	S344	NI	NI
13	Q347	Q347	5
14	Q725	Q725	19
15	F728	F728	13
16	A871	NI	NI
17	G872	NI	NI
18	E875	NI	NI
19	Q946	Q946	1
20	M949	M949	1

21	Y953	Y953	1
22	F983	F983	17
23	M986	M986	5
24	A987	NI	NI
25	Q990	Q990	8

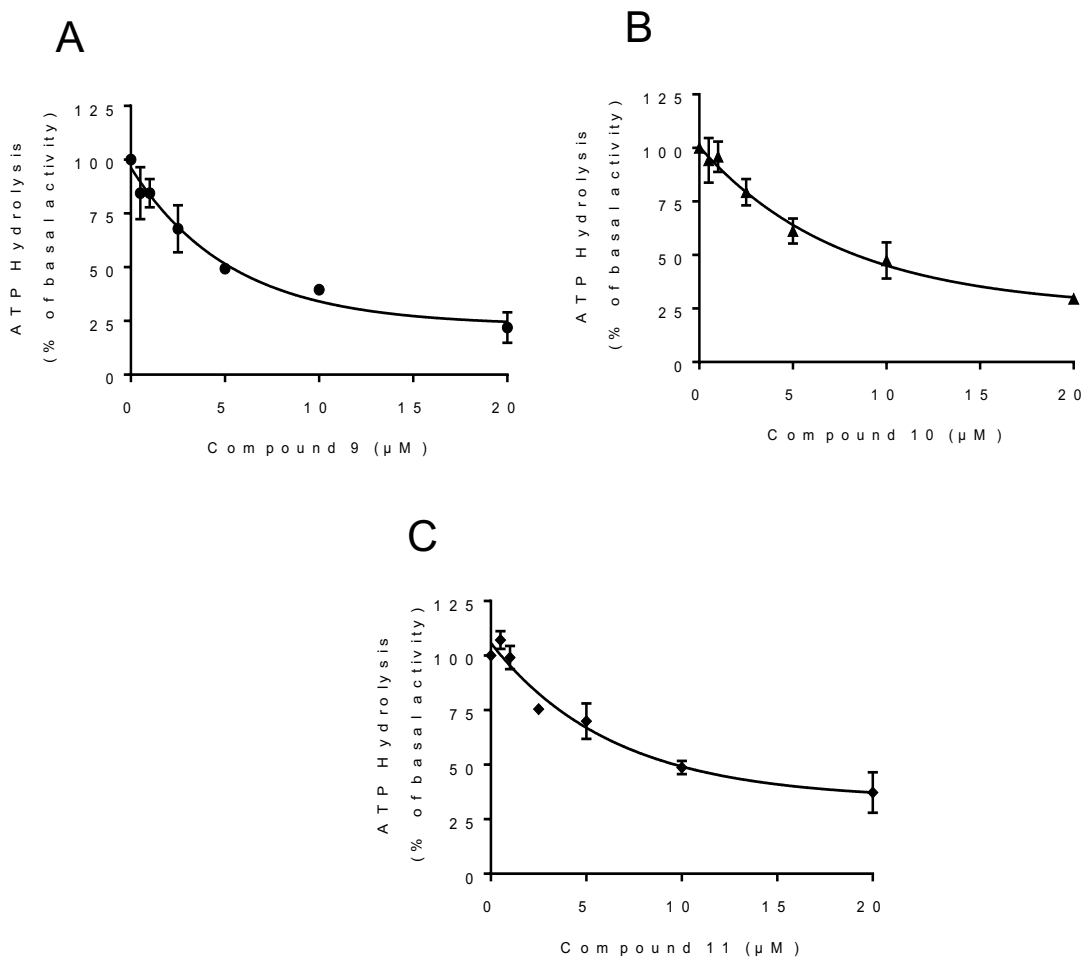
Supplemental Table 1 legend: Comparison of residues that interact with compound 3 and Taxol in human P-gp (pdb.6QEX). The model of the transporter was derived from the recent cryo–electron microscopy determined structure of human P-gp with bound Taxol (Alam et al., 2019) (PDB: 6QEX). The structure of human P-gp bound to Taxol (paclitaxel) revealed that 26 residues in the drug-binding pocket are within 4 Å of Taxol, indicating their interaction with this ligand. Of these 26 residues, 18 were found to interact with compound **3** (number of poses > 5). Residues Y307, Y310, F336, F343, Q725, F728, and F983 were selected for mutagenesis (according to ATPase activity studies) and were found to interact with compound **3** (number of poses>12). NI, no interaction.

Supplemental Table 2

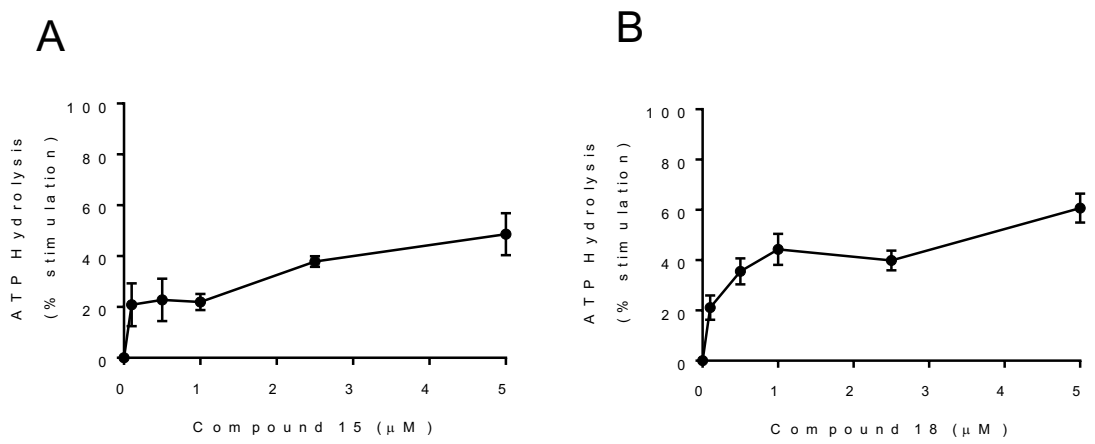
Taxol (Paclitaxel)		Compound 8	
#	Residue	Residue	Number of Poses
0	L65	L65	4
1	M68	NI	NI
2	M69	NI	NI
3	W232	NI	NI
4	F303	F303	20
5	I306	I306	16
6	Y307	Y307	16
7	Y310	Y310	20
8	F336	F336	19
9	L339	L339	14
10	I340	I340	14
11	F343	F343	17
12	S344	S344	4
13	Q347	Q347	7
14	Q725	Q725	20
15	F728	F728	20
16	A871	NI	NI
17	G872	NI	NI
18	E875	NI	NI
19	Q946	Q946	1
20	M949	M949	2

21	Y953	Y953	6
22	F983	F983	20
23	M986	M986	6
24	A987	NI	NI
25	Q990	Q990	11

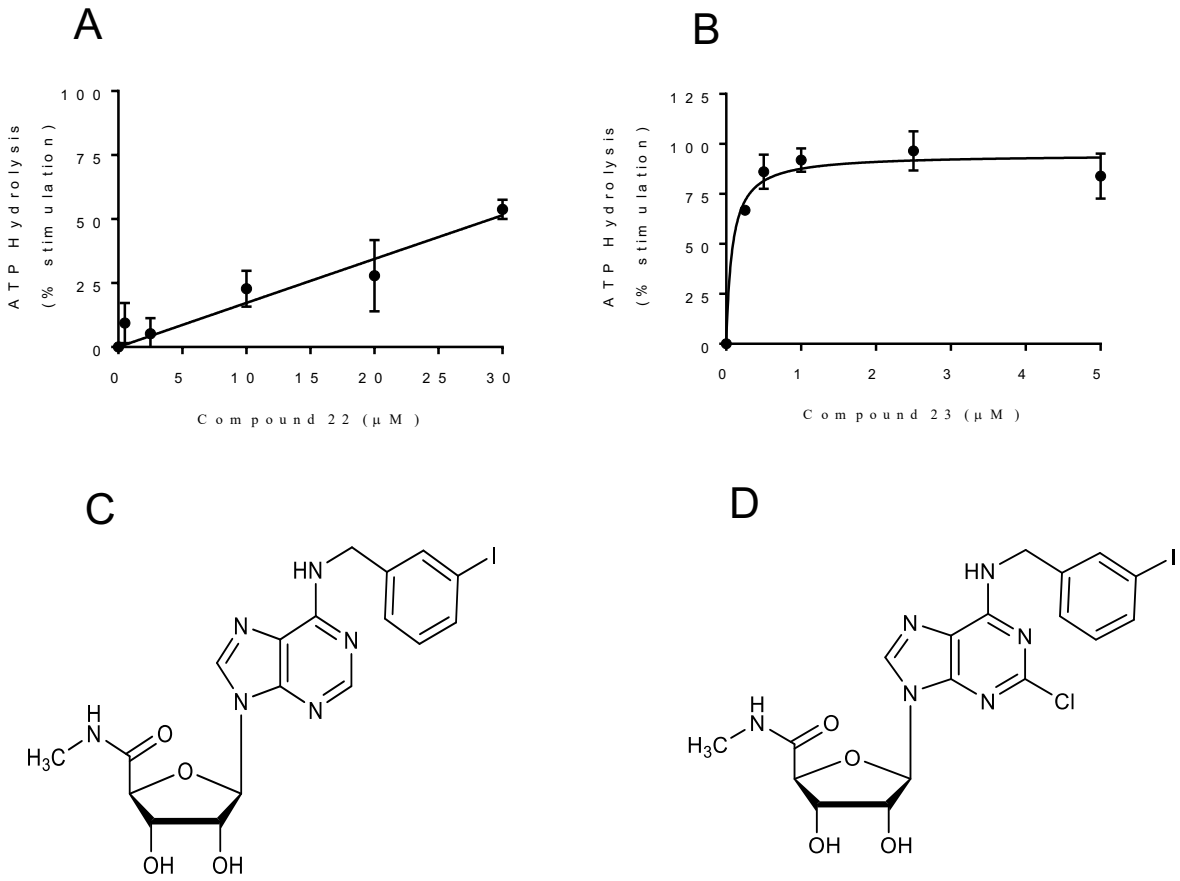
Supplemental Table 2 legend: List of residues interacting with compound 8 and Taxol in human P-gp 6QEX structure. The docking of compound **8** was carried out by the same method used for compound **3** in Table S1. The table shows that 23 out of 26 residues are common for interaction with both compound **8** and Taxol. Residues Y307, Y310, F336, F343, Q725, F728, and F983 were selected for mutagenesis (validated by ATPase activity studies) and were found to interact with compound **8** (number of poses>16). NI, no interaction.



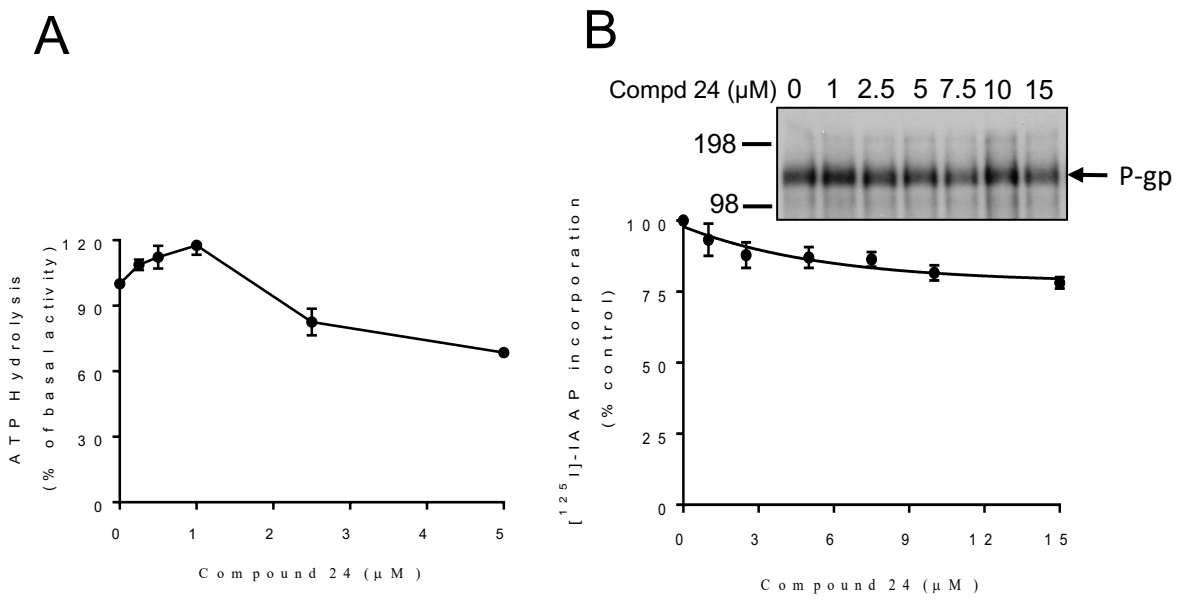
Supplemental Figure 1. Effect of compounds 9 (A), 10 (B) and 11 (C) on the ATPase activity of P-gp. ATPase assay was done as described in the "Materials and Methods section". The curves represent the mean ± SD values from three independent experiments performed in duplicates. GraphPad Prism 7.0 was used to calculate the IC_{50} values given for compound **9** (IC_{50} = 3.69 ± 0.03 μM) (A), **10** (IC_{50} = 5.27 ± 0.02 μM) (B), and **11** (IC_{50} = 4.45 ± 0.03 μM), respectively.



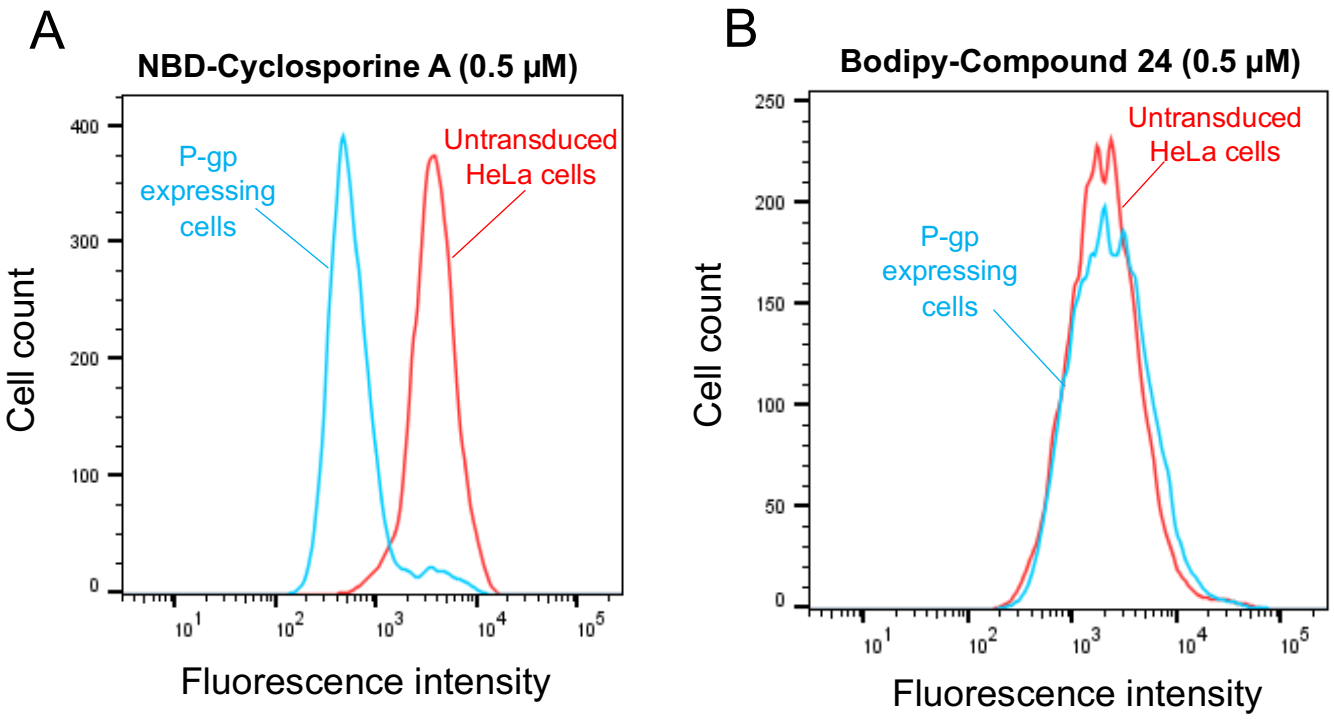
Supplemental Figure 2. Effect of compounds 15 (A) and 18 (B) on the ATPase activity of P-gp. ATPase assay was done using membranes of human P-gp expressing High-Five insect cells, as described in the “Materials and Methods section”. The curves represent the mean ± SD values from three independent experiments performed in duplicates.



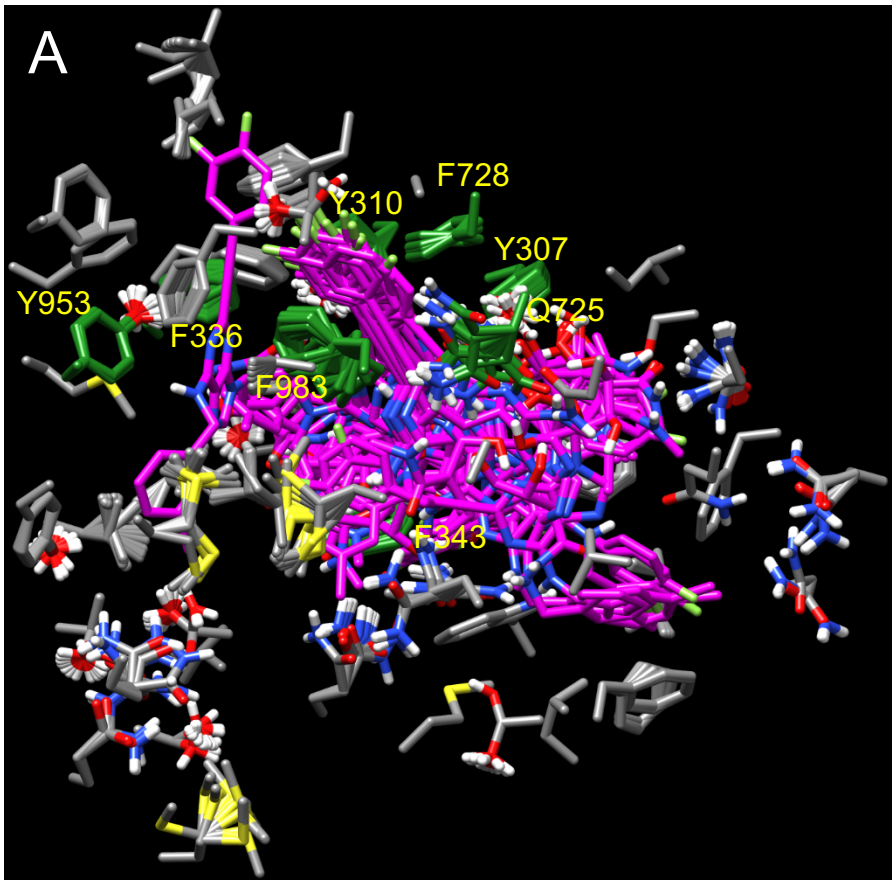
Supplemental Figure 3. Effect of compounds 22 (A) and 23 (B) on the ATPase activity of P-gp. ATPase assays were performed as described in the "Materials and Methods section". The curves represent the mean ± SD values from three independent experiments performed in duplicates. The chemical structure of compound **22** (C) and **23** (D) is shown. The EC_{50} = 0.08 ± 0.002 μM value given in supplemental Figure 3B, was calculated using GraphPad Prism 7.0.

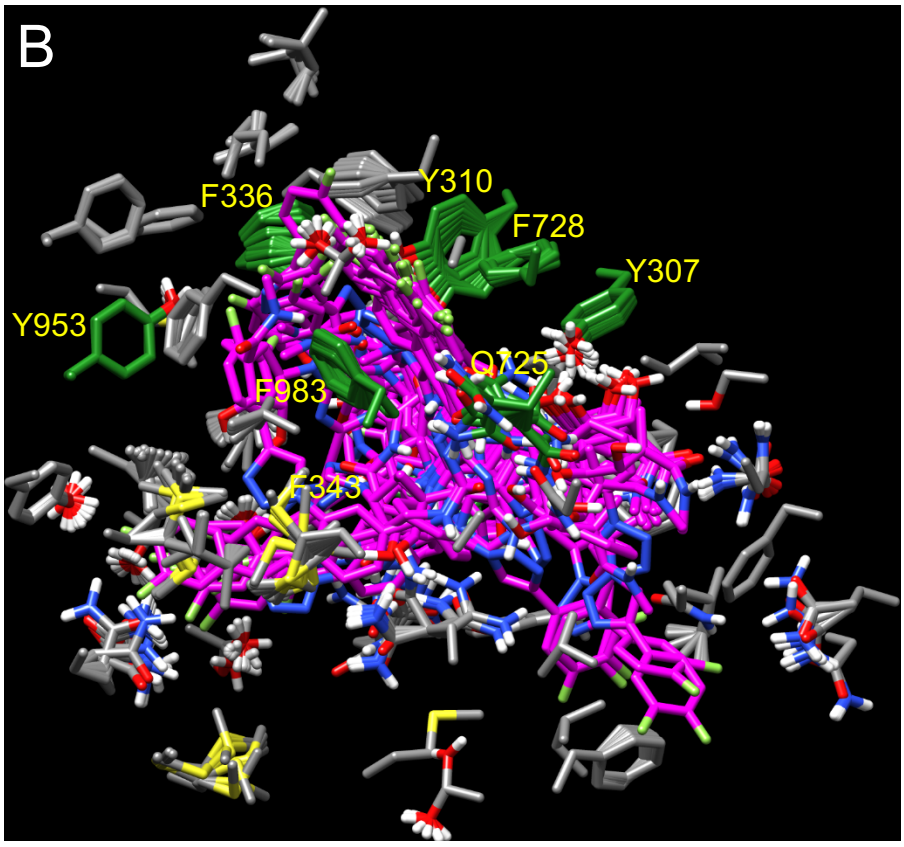


Supplemental Figure 4. The effect of compounds 24 on the ATPase activity of P-gp (A), and the photoaffinity labeling of P-gp by [^{125}I]-iodoarylazidoprazosin (IAAP) (B). The ATPase and photolabeling with IAAP assays were carried out as was described in the "Materials and Methods section". Autoradiograms of IAAP-labeled P-gp bands in the presence of indicated concentrations of compound **24** (panel B) is shown at the top. The position of the molecular weight markers is shown on the left. The curves were plotted using GraphPad Prism 7.0., and plot values were obtained in three independent experiments carried out in duplicate.



Supplemental Figure 5. Bodipy conjugated compound 24 is not transported by P-gp. The histograms show the transport assay for NBD-Cyclosporine A (A) and Compound **24** (B). Human P-gp expressing HeLa cells were incubated with 0.5 µM NBD-cyclosporine A (A) and 0.5 µM of BODIPY-compound **24** (B) for 45 min and their steady-state accumulation in cells was compared. In this assay, untransduced cells were used as a control. Representative histograms show steady-state transport of NBD-cyclosporine A at 0.5 μM (A), but show no transport of compound **24** (B). We also obtained similar results when various concentrations of compound **24** (0.05 μM to 2.5 μM) were used (Data not shown).


A
F728
Y310
Y307
Y953
Q725
F336
F983
F343


B
F336
Y310
F728
Y307
Y953
Q725
F983
F343

Supplemental Figure 6. The cluster of 20 docking poses of Compounds 3 (A) and 8 (B) in the drug-binding pocket of human P-gp structure (pdb: 6QEX) generated by the AutoDock Vina program. The compounds and proximal P-gp residue sidechains are shown as sticks. The color code is: Nitrogen – blue, Oxygen – red, Hydrogen – white, Sulfur – yellow, Fluorine - Chartreuse, and Chlorine – light green. The carbons of the ligands are colored magenta, those of the mutated residues – dark green, and grey for the rest of the residues. The residues selected for mutagenesis are highlighted with yellow font.