Bioconjugate Chemistry

ARTICLE

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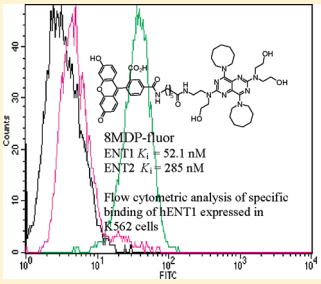
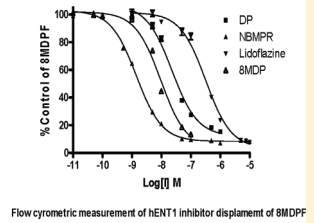
Design, Synthesis, and Evaluation of 2-Diethanolamino-4,8-diheptamethyleneimino-2-(*N*-aminoethyl-*N*-ethanolamino)-6-(*N*,*N*-diethanolamino)pyrimido[5,4-*d*]pyrimidine-fluorescein Conjugate (8MDP-fluor), As a Novel Equilibrative Nucleoside Transporter Probe

Wenwei Lin and John K. Buolamwini*

Department of Pharmaceutical Sciences, College of Pharmacy, 847 Monroe Avenue, Suite 327, University of Tennessee Health Sciences Center, Memphis, Tennessee 38163, United States

ABSTRACT: Nucleoside transporters are integral membrane glycoproteins that play critical roles in physiological nucleoside and nucleobase fluxes, and influence the efficacy of many nucleoside chemotherapy drugs. Fluorescent reporter ligands/substrates have been shown to be useful in the analysis of nucleoside transporter (NT) protein expression and discovery of new NT inhibitors. In this study, we have developed a novel dipyridamole (DP)-based equilibrative nucleoside transporter 1 (ENT1) fluorescent probe. The potent ENT1 and ENT2 inhibitor analogue of dipyridamole, 2,6-bis(diethanolamino)-4,8-diheptamethyleneiminopyrimido[5,4-*d*]pyrimidine (**4**, 8MDP), was modified to replace one β-hydroxyethyl group of the amino substituent at the 2-position with a β-aminoethyl group and then conjugated through the amino group to 6-(fluorescein-5-carboxamido)hexanoyl moiety to obtain a new fluorescent molecule, 2-diethanolamino-4,8-diheptamethyleneimino-2-(*N*-aminoethyl-*N*-ethanolamino)-6-(*N*,*N*-diethanolamino)pyrimido[5,4-d]pyrimidine-fluorescein conjugate, designated 8MDP-fluorescein (8MDP-fluor, **6**). The binding affinities of 8MDP-fluor at ENT1 and ENT2 are reflected by the uridine uptake inhibitory K_i values of 52.1 nM and 285 nM, respectively. 8MDP-fluor was successfully demonstrated to be a flow cytometric probe for ENT1 comparable to the nitrobenzylmercaptopurine riboside (NBMPR) analogue ENT1 fluorescent probe SAENTA-*X*8-fluorescein (SAENTA-fluor, **1**). This is the first reported dipyridamole-based ENT1 fluorescent probe, which adds a novel tool for probing ENT1, and possibly ENT2.





Flow cyrometric measurement of hENT1 inhibitor displamemt of 8MDPF

INTRODUCTION

Specialized integral membrane glycoproteins known as nucleoside transporters (NTs) are required for the influx and/or efflux of physiological nucleosides and many synthetic analogues.[1−3] Nucleoside transporters are classified into two families, namely, the equilibrative nucleoside transporter (ENT) and the concentrative nucleoside transporter (CNT) families, which are also known as the SLC29 and SLC28 gene families, respectively.[4,5] ENTs are the more widely distributed of the two NT families in mammalian tissues and possess broad substrate specificities for both purine and pyrimidine nucleosides, with ENT2 also efficiently transporting nucleobases.

Nucleoside transporters play key roles in nucleoside and nucleobase uptake in salvage nucleotide synthesis and are also critical for the cellular uptake of several nucleoside analogues used in the treatment of cancers and viral infections, and serve as biomarkers of drug efficacy and drug resistance.[1,6,7] In addition, by regulating the concentration of adenosine available to cell surface adenosine receptors, they influence physiological processes such as cardiovascular activity, neurotransmission, and kidney function among others.[3,8−10] Distinct regional distributions of ENT1 and ENT2 have been observed in the human central nervous system.[11] It has also recently been shown that ENT1 and ENT2 play crucial roles in regulating the cystic fibrosis-related chloride transporter (CFTR) in human airway epithelia.[12] The inhibition of adenosine transport by NT inhibitors can potentially be exploited for site- and event-specific therapeutic approaches to treating ischemic heart disease and stroke.[13−18] Specific/selective inhibitors and ligands bound with high affinity are needed to track and quantify NT protein expression as well as to investigate their physiological roles and explore their therapeutic potential.

In the evaluation of nucleoside transporter inhibitors, radioligand uptake/displacement experiments have been widely used since nonradioactive methods are largely unavailable. Fluorescent probes are now available for ENT1,[19−21] but there are no such probes for other ENTs or CNTs. The fluorescent probes allow ligand−cell/protein interactions to be analyzed by flow cytometry, a facile technique that can eliminate problems associated with

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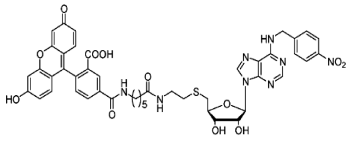
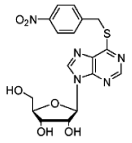
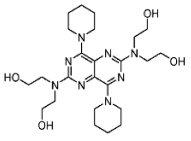
the use of radioactive materials. 5-(SAENTA)-*X*8-fluorescein (SAENTA-fluor, SF, **1**) is an example of an ENT1 fluorescent probe.[20] As an analogue of nitrobenzylmercaptopurine riboside (NBMPR, **2**), it has been used in several studies for nonradioactive assays of compounds' ENT1 inhibitory activities.[22−26]



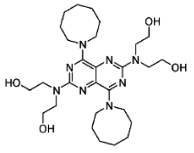
1 (SAENTA-*X*8-fluorescein, SF)



2 (NBMPR)



3 (Dipyridamole, DP)



4 (Heptamethyleneimine DP analog, 8MDP)

In our attempts to develop new ENT fluorescent probes, we have concentrated on the best compound we identified from a structure−activity relationship study of a series of dipyridamole (DP, **3**) analogues that we synthesized recently as ENT1 and ENT2 inhibitors.[23,27] This compound, the 8-membered ring dipyridamole analogue, compound **4** (8MDP) had K_i values of 0.97 nM and 90.8 nM against hENT1 and hENT2, respectively,[27] indicating much higher binding affinities than DP, which had K_i values of 14.5 nM and 308 nM against hENT1 and hENT2, respectively.[27] In the current study, we attached a fluorescent reporter group to a modified 8MDP to obtain a new fluorescent probe, which was successfully used in flow cytometric analysis of ENT1 protein expression as well as the measurement of relative affinities of ENT1 ligands.

EXPERIMENTAL PROCEDURES

Chemistry. Thin-layer chromatography (TLC) was conducted on silica gel plates (Analtech). Compounds were visualized by UV light (254 and 365 nm). 1D NMR spectra were recorded on a Varian Inova 500 MHz NMR instrument, using $CDCl_3$ or $(CD_3)_2SO$ as solvents and tetramethylsilane (TMS) as an internal standard. Flash column chromatography was performed on Fisher silica gel (170−400 mesh). Melting points were determined using a Fisher-Johns melting point apparatus and were reported uncorrected. Mass spectra were obtained on a Bruker-HP ESQUIRE Ion Trap LC/MS system. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA. All solvents and reagents were purchased from Aldrich or other major chemical companies and used without further purification. All reactions were carried out under argon gas. Analytic high performance liquid chromatography (HPLC) was performed on a 150 mm × 2.00 mm Phenomenex Luna C_{18} column (3-μm particle size), protected by a Phenomenex SecurityGuard guard column (cartridge 4 mm × 2.00 mm internal diameter); and gradient CH_3CN/H_2O; 0 to 100 (15 min) with detection at 224 nm. The HPLC system consisted of an automated system with two Shimadzu LC-10AD VP liquid chromatography pumps, an SCL-10A VP system controller, a SIL-10AD VP auto injector, a sample cooler kept at 4 °C, and an SPD-M10A VP diode array detector. Chromatograms were acquired and analyzed with the Schimadzu Class-vp 7.3 SP1 software running on a Dell computer.

*2-Diethanolamino-4,8-diheptamethyleneimino-6-chloropyrimido[5,4-d]pyrimidine (**6**).* To a solution of 2,4,6,8-tetrachloropyrimido[5,4-*d*]pyrimidine (TCPP, **5**) (0.54 g, 2 mmol) in anhydrous THF (20 mL), heptamethyleneimine (1.06 mL, 8.4 mmol) was added. The reaction was stirred in an ice−water bath for 20 min, and then water (150 mL) was added to precipitate the reaction intermediate. After drying over a P_2O_5 desiccator, the intermediate was dissolved in dimethylsulfoxide (DMSO) (10 mL) with diethanolamine (3 mL, 30 mmol). The reaction was stirred at 120 °C for 3 days. The product, compound **6** (445 mg, 45% overall yield in 2 steps), was purified by flash silica gel chromatography (hexane/acetone = 5/1). Mp: 122−124 °C; MS (ESI) *m*/*z* 492 $(M + H)^+$, 514 $(M + Na)^+$. ^{1}H NMR (DMSO-d_6) δ 4.737 (t, 2H, exchanged with D_2O, 2 × OH, *J* = 5 Hz), 3.868 (m, 8H, 2 × $N(CH_2CH_2CH_2)_2CH_2$), 3.589 (t, 8H, $N(CH_2CH_2OH)_2$, *J* = 5 Hz), 1.796 (s, 8H, 2 × $N(CH_2CH_2CH_2)_2CH_2$), 1.534 (s, 8H, 2 × $N(CH_2CH_2CH_2)_2CH_2$), 1.495 (s, 4H, 2 × $N(CH_2CH_2CH_2)_2CH_2$). Anal. ($C_{24}H_{38}ClN_7O_2$) C, H, N.

*2-Diethanolamino-4,8-diheptamethyleneimino-6-[(N-dibenzylaminoethyl)-ethanolamino]-pyrimido[5,4-d]pyrimidine (**7**).* Compound **6** (370 mg, 0.75 mmol) was heated with 2-(2-dibenzylamino-ethylamino)-ethanol (3 g, 10 mmol) at 150 °C overnight. The product, compound **7** (130 mg, 23%), was purified by flash silica gel chromatography (hexane/acetone = 2/1) as a yellow sticky solid. MS (ESI) *m*/*z* 762 $(M + Na)^+$. ^{1}H NMR (DMSO-d_6) δ 7.306 - 7.251 (m, 10H, Ar), 7.181 (m, 4H, 2 × CH_2), 4.698 (s, 2H, disappeared after addition of D_2O, 2 × OH), 4.606 (s, 1H, disappeared after addition of D_2O, OH), 4.007−3.677 (*br* s, 8H, 2 × $N(CH_2CH_2CH_2)_2CH_2$), 3.575 (s, 12H, $N(CH_2CH_2OH)_2$, NCH_2CH_2OH), 3.520 (d, 4H, NCH_2CH_2ON, *J* = 6.5 Hz), 1.726 (*br* s, 8H, 2 × $N(CH_2CH_2CH_2)_2CH_2$), 1.496 (*br* d, 12H, 2 × $N(CH_2CH_2CH_2)_2CH_2$). LC retention time 5.804 min.

*2-Diethanolamino-4,8-diheptamethyleneimino-6-[(N-6-(fluorescein-6-carboxamido)-hexanoylaminoethyl)- ethanolamino]-pyrimido[5,4-d]pyrimidine (**8**).* The benzyl groups on compound **7** (100 mg, 0.13 mmol) were removed by hydrogenation in the presence of a catalytic amount of 10% Pd−C in methanol. After filtration and evaporation, the residue was reacted with commercially available 6-(fluorescein-5-carboxamido)hexanoic acid succinimidyl ester (10 mg, 17 μmol) in dimethylformamide (DMF) (3 mL) for 2 h. The solvent was removed under reduced pressure, and the residue was purified by sephadex LH-20 gel filtration with MeOH as the elution solvent to obtain the product **8** (7.2 mg, 41%) as a sticky gum. MS (ESI) *m*/*z* 1029 $(M - H)^-$. LC retention time 11.368 min.

Biological Testing. *[^{3}H]Uridine Uptake Experiments with K562 Cells Used to Determine ENT1 Inhibitory Activities of Test Compounds.* Human chronic myelogenous leukemia K562 cells (American Type Culture Collection (ATCC), Manassas, VA) were maintained in RPMI 1640 medium containing 10% fetal bovine serum and amikacin (60 mg/Liter), at 37 °C in 5% CO_2 and 95% air atmosphere. All experiments were done in sodium free uptake buffer (20 mM Tris/HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2$, 5 mM glucose, and 130 mM *N*-methyl-D-glucamine/HCl, pH 7.4). 5×10^5 K562 cells/sample were harvested and washed once with sodium free uptake buffer and resuspended in 50 μL of the same buffer. Uptake buffer

containing varying concentrations of compounds was then added, followed by 15 min of incubation, and then [^{3}H]uridine (37 mCi/mL) at a final concentration of 0.5 μM was added, followed by 2 min of incubation. Uptake was stopped by fast filtration to remove extracellular radioactive material, followed by 5 rapid washes with ice-cold uptake buffer. Cells were then solubilized overnight in 0.5 mL of 5% Triton X-100, and radioactivity was measured by β-scintillation counting.

[^{3}H]Uridine Uptake Experiments with PK15 Cells Stably Expressing Recombinant hENT2 Protein. The ENT2 cell line (porcine PK15 cells stably transfected with recombinant human ENT1 (hENT1)[28] was generously provided by Dr. Chung-Ming Tse, Johns Hopkins University. Cells were maintained in minimum essential (MEM) Alpha medium, with 5% fetal bovine serum, penicillin/streptomycin (50,000 units/L, 50 mg/L), and 0.1 mM nonessential amino acids, at 37 °C with 5% CO_2 and 95% air mixture. Cells were seeded at 40,000 cells/well in 48-well plates 24 h prior to uptake experiments. All experiments were performed at room temperature in sodium-free uptake buffer (140 mM *N*-methyl-D-glucamine, 5 mM HEPES, 5 mM KH_2PO_4, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM glucose, pH 7.4). Cells at 60−80% confluency were washed 3 times with uptake buffer, followed by 10 min of preincubation in the same buffer. Solutions containing varying concentrations of test compounds in uptake buffer were then added, followed by 15 min of incubation and addition of [^{3}H]uridine (0.2 μM, 37 mCi/mL specific activity). After 2 min of incubation with [^{3}H]uridine, cells were rapidly washed 3 times with ice-cold phosphate-buffered saline (pH 7.4) to stop uptake and remove extracellular [^{3}H]uridine. The cells were then solubilized overnight in 0.5 mL of 5% Triton X-100, and radioactivity was measured by a β-scintillation counter.

Uptake Inhibition Data Analysis. Percentage (%) inhibition was calculated for each sample by eq 1.

$$\%\ \text{inhibition} = 100\% - \frac{([^3\text{H}]\text{uridine}_s) \times 100\%}{([^3\text{H}]\text{uridine})} \quad (1)$$

where [^{3}H]uridine$_s$ is the amount of [^{3}H]uridine transported by hENT1 (or hENT2) in the presence of the test compound, and [^{3}H]uridine is the amount of [^{3}H]uridine transported in the absence of the test compound. The results were fed into the PRISM program (GraphPad, San Diego, CA) to derive concentration-inhibition curves. From these curves, the IC_{50} values were obtained and used to calculate the inhibition constant (K_i) value by eq 2.[29]

$$K_i = \text{IC}_{50}/(1 + [L]/K_L) \quad (2)$$

where [L] is the concentration of [^{3}H]uridine in individual tests (0.5 μM for hENT1; 0.2 μM for hENT2), and K_L is the K_m values for the uptake of [^{3}H]uridine by the corresponding transporter, 229 μM for hENT1 and 250 μM for hENT2.[28] The K_i values were used to compare the abilities of compounds to inhibit [^{3}H]uridine uptake and, for that matter, their inhibitory activity against hENT1 (or hENT2).

Flow Cytometry Assays. The compounds were tested to determine their ENT1 binding ability by a flow cytometric assay.[22] Briefly, K562 cells growing in RPMI 1640 medium were washed once, resuspended at 1.6×10^6 cells/mL in phosphate-buffered saline at pH 7.4, and incubated with SAENTA-fluorescein (30 nM) or 8MDP-fluor (40 nM) in the presence or absence of varying concentrations of test compounds at room temperature for 45 min. Flow cytometric measurements of cell-associated fluorescence were then performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 mW-argon laser (Molecular Resources Flow Cytometry Facility, University of Tennessee Health Sciences Center). In each assay, 5,000 cells were analyzed from suspensions of 4×10^5 cells/mL. The units of fluorescence were arbitrary channel numbers. Percentage (%) inhibition (i.e., ENT1-specific fluorescence in the presence of SAENTA-fluorescein or 8MDP-fluor) was calculated for each sample by eq 3.

$$\%\ \text{inhibition} = 100\% - \frac{(F_s) \times 100\%}{(F_f)} \quad (3)$$

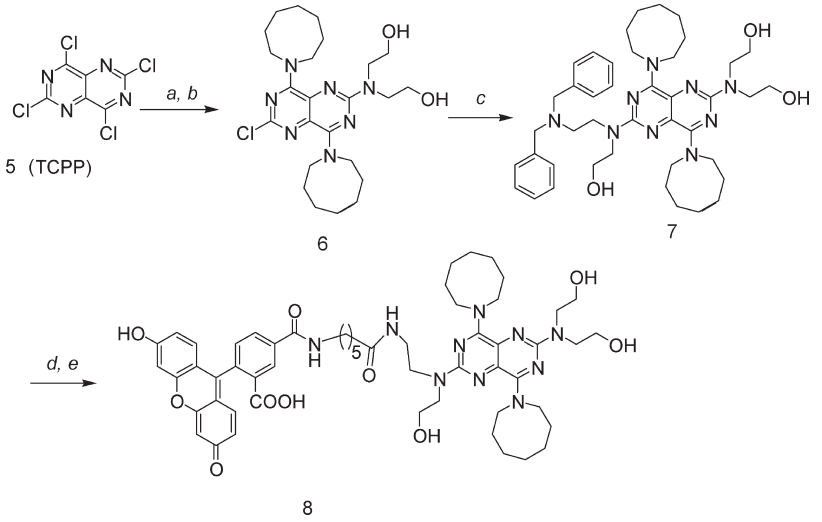
where F_s is the ENT1-specific fluorescence in the presence of test compounds, and F_f is the ENT1-specific fluorescence without the test compound, in mean channel numbers. The results were used to derive concentration-inhibition curves (Prism program) for IC_{50} values, which were used to calculate K_i values using eq 2, where [L] and K_L are the concentrations of the fluorescent probe (30 nM for SAENTA-fluor and 40 nM for 8MDP-fluor) and K_d values of the fluorescent probes (1.8 nM for SAENTA-fluor and 52.1 nM for 8MDP-fluor), respectively. The K_i values were used to compare the affinities of compounds for hENT1.

RESULTS

Chemistry. The synthetic route we followed, Scheme 1, resulted in the successful preparation of the new fluorescent probe. Starting with the reaction between 2,4,6,8-tetrachloropyrimido[5,4-*d*]pyrimidine (TCPP, **5**) with excess heptamethyleneimine (4.2-fold ratio) at 0 °C gave the 2,6-dichloro-4,8-diheptamethyleneimido intermediate. Reaction of the 2,6-dichloro intermediate with one equivalent of diethanolamine at 120 °C yielded the monochloro precursor compound **6** in overall yield of 45% for the two steps. Compound **6** was in turn reacted with 2-(2-dibenzylaminoethylamino)-ethanol at 150 °C to produce the protected amine compound, **7**. Removal of the benzyl groups of compound **7** was successfully effected by catalytic hydrogenation, which was followed by the reaction of the resulting primary amine with the activated fluorescein derivative, 6-(fluorescein-5-carboxamido)hexanoic acid succinimidyl ester to afford the target fluorescent probe, 8MDP-fluorescein conjugate, 8MDP-*X*8-fluorescein (8MDP-fluor, **8**) in 43% yield overall from the latter two steps.

Biology. *ENT1 and ENT2 Inhibitory Activities and 8MPD-Fluor.* For a probe to be useful, it must be bound by its intended molecular target(s) with high affinity, in this case ENT1 and ENT2. To assess this for the new fluorescent probe, we measured its ability to inhibit [^{3}H]uridine uptake by hENT1 expressed in K562 cells or recombinant hENT2 stably expressed in K15NTD cells. Dose−response experiments were carried out, and the IC_{50} values were used to derive inhibition constant (K_i) values according to eq 2, which are presented in Table 1 along with the K_i values of known equilibrative nucleoside transporter inhibitors that were also determined by the same methods, including dipyridamole and 8MDP (**4**, the most potent dipyridamole analogue ENT inhibitor reported to date). The results show that 8MDP-fluor (**8**) exhibited a reasonably high-affinity binding by hENT1 with a K_i value of 52.1 nM and a moderate-affinity binding by hENT2, with a K_i value of 285 nM, which is reasonable, considering that dipyridamole, the prototype ENT2 inhibitor, exhibited an ENT2 inhibitory K_i of 308 nM.

Scheme 1. Synthesis of 8MDP-Fluor (8)[a]



[a] Reagents: (a) heptamethyleneimine, anhydrous THF, ice−water bath; (b) diethanolamine, DMF, 120 °C; (c) 2-(2-dibenzylamino-ethylamino)-ethanol,[27] DMSO, 150 °C; (d) hydrogen, 10% Pd−C, MeOH; (e) 6-(fluorescein-5-carboxamido)hexanoic acid succinimidyl ester, DMF.

Table 1. hENT1 and hENT2 Inhibitory Activities of 8MPD, Its fluorescent Analogue 8MPD-F and Other Inhibitors

	hENT1 inhibitory activity		hENT2 inhibitory activity	
compound	% inhibition (at 10 μM)	K_i (nM)	% inhibition (at 10 μM)	K_i (nM)
DMSO	2.8 ± 0.1	NA[a]	0.0 ± 1.8	NA
9 (lidoflazine)	81.1 ± 0.1	573.8	43.2 ± 1.0	12,390
2 (NBMPR)	94.1 ± 0.1	0.65	90.1 ± 0.1	1,349
3 (dipyridamole)	97.4 ± 0.1	15.1	98.2 ± 0.2	308
4 (8MDP)	97.6 ± 0.1	0.97	98.9 ± 0.1	90.7
8 (8MDP-fluor)	99.6 ± 0.2	52.1	93.9 ± 0.1	285
1 (SAENTA-fluor)	92.6 ± 0.8	1.8	83.5 ± 0.4	4,436

[a] NA means not applicable.

8MDP-Fluor Exhibits Specific Staining of hENT1. To assess the effectiveness of 8MDP-fluor as a fluorescent probe, we compared it with SAENTA-fluorescein, which is used in flow cytometric analysis of ENT1 protein expression and testing of ENT1 ligands in our laboratory and by others.[22,26,30] Again, human CML K562 cells were used since they grow in suspension culture, which makes them highly suitable for use in flow cytometry experiments, unlike attached cells which have to be first detached and suspended for flow cytometry. As shown in Figure 1, the new 8DMP-fluor stained K562 cells in an ENT1-specific manner comparable to that by SAENTA-fluor. It took 40 nM concentration of 8DMP-fluor to obtain ENT1 staining comparable to that by 30 nM of SAENTA-fluor. This reflects the lower ENT1 binding affinity for 8MDP-F compared to that of SAENTA-fluor, as evident from Table 1. The interesting thing is that the higher concentration of 8DMP-fluor did not result in higher nonspecific staining as shown by staining in the presence of the standard ENT1 inhibitor NBMPR.

Comparison of 8MDP-Fluor and SAENTA-Fluor As Fluorescent Ligands for Use in Flow Cytometric Determination of the Relative Affinities of ENT1 Interacting Compounds. To test the potential utility of this new fluorescent molecule (8MDP-fluor) as a probe for assessing other molecules' ability to bind to ENT1, NBMPR, lidoflazine, dipyridamole, and 8MDP were used to displace 8MDP-fluor (8MDPF) from K562 cells in a concentration-dependent manner in flow cytometry experiments. For comparison, the same tests were performed with SAENTA-fluor (SF) as the fluorescent ligand. Radioligand binding studies have indicated that NBMPR, dipyridamole, and lidoflazine displace each other from ENT1.[31−33] Dipyridamole itself is fluorescent, with $Ex_{\lambda max}$ and $Em_{\lambda max}$ of 280 and 490 nm, respectively.[34] At the experimental dipyridamole concentrations and wavelengths used in the flow cytometric assay (Ex_{λ} = 488 nm and Em_{λ} = 533 nm), dipyridamole and 8MDP did not emit fluorescence that would interfere with the detection of bound 8MDP-fluor or SAENTA-fluor.

The dose-response displacement curves are shown in Figure 2, and the K_i values derived from them are presented in Table 2. Flow cytometric experiments using 8MDP-fluor as probe afforded K_i values of 0.81, 5.41, 12.9, and 181 nM for NBMPR, 8MDP, dipyridamole, and lidoflazine (**9**), respectively, whereas experiments that used SAENTA-fluor as the probe afforded K_i values of 0.43, 0.50, 8.18, and 279.9 nM, for NBMPR, 8MDP, dipyridamole and lidoflazine, respectively. Although the use of either the 8MDP-fluor or SAENTA-fluor as the probe indicated correctly the same relative order of ENT1 binding affinities for the inhibitors, it

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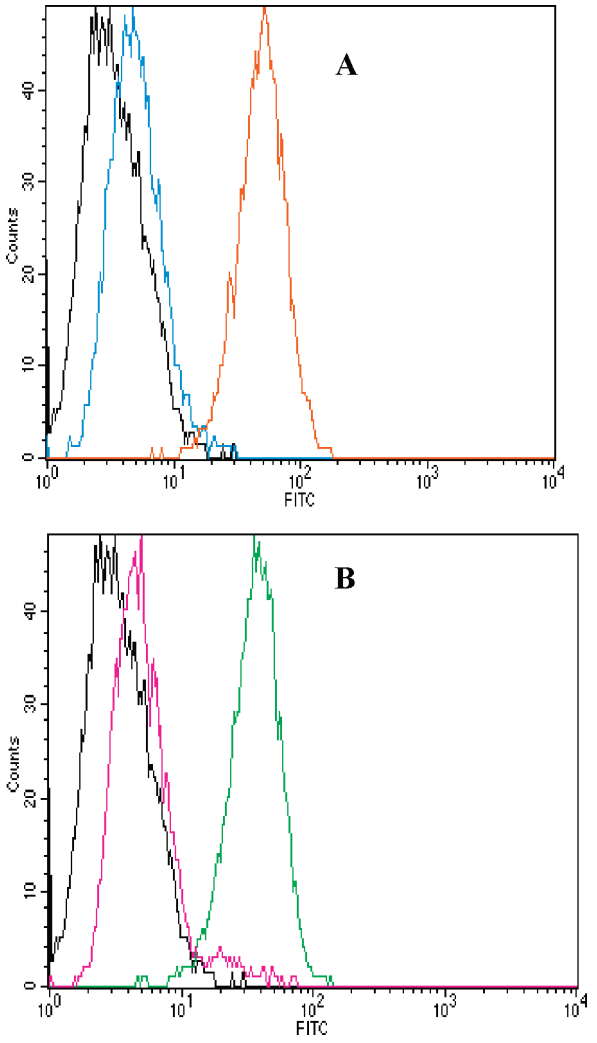
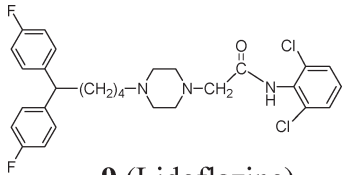


Figure 1. Flow cytometric histograms showing the ENT1 specific labeling in K562 leukemic cells by 8MDP-fluor (8MDPF, 40 nM; panel A) or SAENTA-fluorescein (SF, 30 nM; panel B). The binding of both fluorescent probes was effectively reversed by the presence of 10 μM NBMPR, the prototype ENT1 inhibitor, showing ENT1 affinity for the probes. Color-coding legend of histograms: black, K562 cells' autofluorescence; orange, K562 cells + 30 nM SF; blue, 30 nM SF + K562 cells +10 μM NBMPR; green line, K562 cells + 40 nM 8MDP-F; and red, K562 cells + 40 nM 8MDP-F + 10 μM NBMPR.

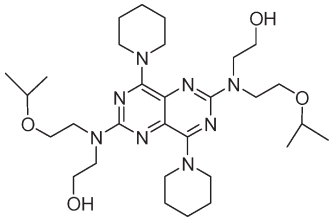
can be seen that the absolute K_i values differed depending on whether 8MDP-fluor or SAENTA-fluor was the fluorescent ligand.



9 (Lidoflazine)

DISCUSSION

Dipyridamole (DP, **3**) has displayed potent inhibitory activity against ENT1 (although not as good as NBMPR) and moderate inhibitory activity against ENT2.[35] It thus provides a good template for optimization to obtain more potent ENT1 and ENT2 inhibitors and probes since it is a clinically used drug. In the course of our structure−activity relationship (SAR) studies of DP analogues, we discovered that replacement of one each of the hydroxyl groups on each diethanolamine substituent at the 2- and 6-positions to give compound **6** had no detrimental effect on ENT1 binding activity but rather enhanced the binding affinity.[23] The high potency of compound **10** (ENT1 K_i = 4.3 nM; ENT2 K_i = 175 nM),[23,27] in which two of the hydroxyl groups of dipyridamole had been replaced by isopropoxy groups, suggested to us that the hydroxyl groups of dipyridamole provide possible attachment positions for reporter groups such as fluorophores. Thus, we took advantage of this opportunity to append a fluorescein group to the most potent dipyridamole analogue ENT1 and ENT2 inhibitor identified to date, compound **4** (8MPD).[23,27] To make the linkage resistant to hydrolysis, one of the hydroxyl groups of compound **4** was replaced by an amino group, which also provided an excellent nucleophilic handle (Scheme 1). The target probe, designated 8MDP-fluorescein conjugate (8MDP-fluor, **8**) was designed to attach the same fluorescent group used to synthesize SAENTA-fluorescein (**1**).[19,20]



10

In the synthesis, the key to achieving selective and sequential substitution of the different chlorines on the starting material, TCPP, was the control of reaction temperature, time, and reactant stoichiometries. The 4- or 8-position chlorine substituents are easier to substitute than the 2- and 6-position chlorines and were replaced at the low temperature of 0 °C, with the sparing of the 2- and 6-position chlorine substituents.

As might be expected, the attachment of a fluorescein moiety and replacement of O with NH in the new fluorescent probe (8MDP-fluor, **8**) significantly lowered inhibitory activities against hENT1 and hENT2, compared to that of the parent compound, 8MDP (**4**), but the fluorescent compound still retained enough potency to serve as an effective probe for ENT1. Interestingly, it still retained higher potency than dipyridamole as an ENT2 ligand and thus could potentially be developed as an ENT2 probe as well. This is not the case with SAENTA-fluor, which hENT2 binds with much lower affinity (K_i = 4,436 nM). Thus, unlike the 8MDP-fluor, SAENTA-fluor can serve as an effective hENT1 probe but not a probe for hENT2.

The results of the flow cytometric determination of the ability of other ENT1 inhibitors to displace 8MDP-fluor or SAENTA-fluor not only showed that the two fluorescent probes can both be used to correctly rank-order the relative affinities of ENT1 ligands but also showed interestingly that it was generally easier to displace SAENTA-fluor from hENT1 than it was to displace the new 8MDP-fluor. This could be due to the larger size of 8MDP-fluor relative to SAENTA-fluor. The only hENT1 inhibitor that deviated from this generalization was lidoflazine (**9**). The reason for this is not apparent, but might be related to possible different binding kinetics of the two fluorescent ligands and/or differences in their binding modes or sites relative to

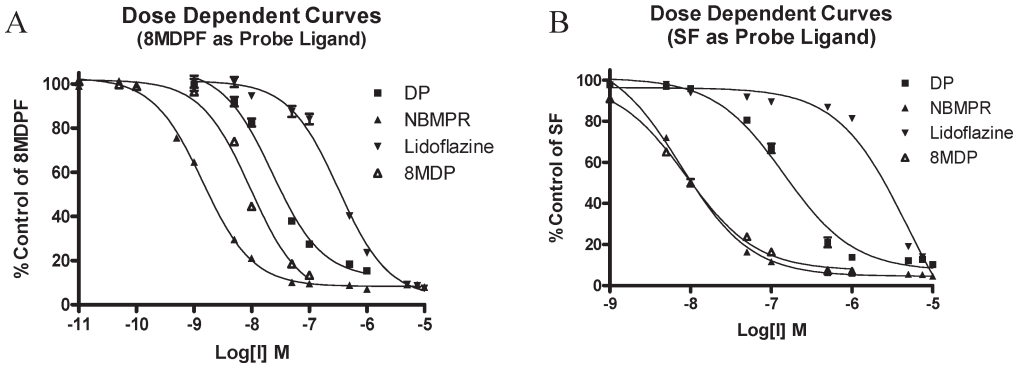


Figure 2. Dose-inhibition curves for the inhibition of 8MDP-fluor (A) or SAENTA-fluor (B) binding to hENT1 determined by a flow cytometric assay using K562 leukemic cells.

Table 2. Use of 8MDP-Fluor and SAENTA-Fluorescein As Ligands for Flow Cytometric Determination of Binding of Standard Inhibitors to hENT1 in K562 Cells

	8MDP-fluor as probe	SAENTA-fluor as probe
compound	K_i (nM)	K_i (nM)
9 (lidoflazine)	182	280
2 (NBMPR)	0.81	0.43
3 (dipyridamole, DP)	12.9	8.18
4 (8MDP)	5.41	0.50

lidoflazine binding. This observation might be worth a follow-up study.

In conclusion, we have synthesized and evaluated a new dipyridamole analogue-based fluorescent probe, which is a high-affinity ligand of both ENT1 and ENT2. We have demonstrated its ability to be an effective flow cytometry probe for the analysis of hENT1 protein expression and for the assessment of relative tightness of binding of compounds that interact with hENT1. Its significant binding to hENT2 suggests that it also has the potential to be used as an ENT2 fluorescent probe.

AUTHOR INFORMATION

Corresponding Author

*Phone: (901) 448-7533. Fax: (901) 448-6828. E-mail: jbuolamwini@uthsc.edu.

Notes

We have no conflicts of interest to report.

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