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Novel halogenated nitrobenzylthioinosine analogs as *es* nucleoside transporter inhibitors

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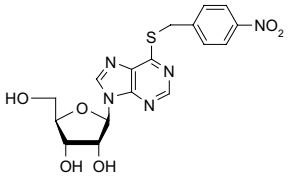
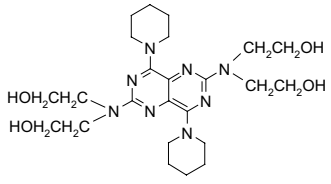
Abstract—Nucleoside transporter inhibitors have potential therapeutic applications as anticancer, antiviral, cardioprotective, and neuroprotective agents. We have synthesized and flow cytometrically evaluated the binding affinity of a series of novel halogenated nitrobenzylthioinosine analogs at the human *es* nucleoside transporter. Structure–activity relationships indicate the importance of hydrophobicity and electron withdrawing capacity of substituents at the *para*-position of the 6-position benzyl substituent. All of the compounds showed high binding affinity as shown by their ability to displace the fluorescent *es* transporter ligand, SAENTA-X8-fluorescein. Compound **16** (6-*S*-(*para*-iodobenzyl)-6-thioinosine) was the most tightly bound within the series with a K_i of 3.88 nM (NBMPR exhibited a K_i of 0.70 nM). This compound has higher affinity than the widely used nonnucleoside, nucleoside transport inhibitor, dipyridamole ($K_i = 8.79$ nM), and may serve as a new lead compound.

Nucleoside transporters are integral membrane glycoproteins that regulate the uptake of physiological nucleosides and their synthetic analogs in cells.[1,2] The two major families of nucleoside transporters are the equilibrative transporters (ENTs) that are ubiquitously distributed in mammalian tissues, and the concentrative sodium-ion linked transporters (CNTs), which are especially abundant in the intestine, kidney, liver, and certain cultured cell lines. Two main equilibrative transporters have been characterized, the *es* (*e*quilibrative *s*ensitive) nucleoside transporter, also known as ENT1 and the *ei* (*e*quilibrative *i*nsensitive) nucleoside transporter, also known as ENT2. These two transporters differ on the basis of their sensitivity or insensitivity to inhibition by S^6-(4-nitrobenzyl)-mercaptopurine riboside (NBMPR, **1**), respectively. However, both of them are inhibited by the nonnucleoside, dipyridamole (**2**). Both NBMPR and dipyridamole are known to inhibit the *es* nucleoside transporter at low nanomolar concentrations.[3] Two new equilibrative transporters, known as ENT3 and ENT4 has been recently identified but not fully characterized.[4,5]



NBMPR, **1**



Dipyridamole, **2**

Nucleoside transporter inhibitors have been shown to have potential therapeutic applications in antimetabolite chemotherapy in cancer, viral infections, and AIDS-related opportunistic infections such as *Toxoplasma gondii*,[3] in inflammatory disease[6] and as cardioprotective and neuroprotective agents.[7–11] Studies involving cellular uptake and efficacy of anticancer and antiHIV nucleoside analogs has also generated interest in nucleoside transporters. The ubiquitously distributed *es* transporter appears as the most important nucleoside transporter of most mammalian tissues and hence it might be the most relevant therapeutic target. Toxicity, lack of in vivo efficacy and/or lack of selectivity have hampered the therapeutic application of the currently available *es* nucleoside transporter inhibitors.[3] Hence, there is a need for developing novel nucleoside transporter inhibitors.

Quite a few substituents on the S^6-benzyl position of NBMPR have been studied.[12] However, largely missing

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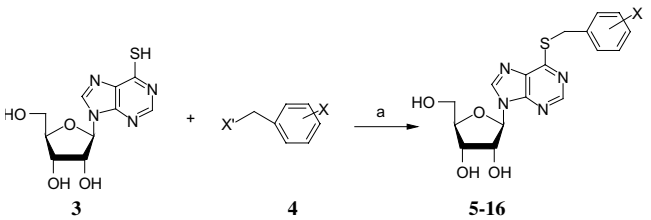
from this group of substituents, are the halogens. This paper reports the synthesis and structure–activity relationship (SAR) studies of new halogen-substituted NBMPR analogs as *es* transporter ligands. The experiments were carried out in order to study the *es* nucleoside transporter binding affinity of the 6-*S*-(*ortho*, *meta*, or *para*-position halogen substituted benzyl) thioinosine. A systematic substitution involving the four halogens, fluorine, chlorine, bromine, and iodine was carried out. A comparison was made between these new halogen analogs and the *es* nucleoside transporter inhibitors, NBMPR, and dipyridamole.

Although none of the compounds appears to be as active as NBMPR ($K_d = 0.1$–1.0 nM), quite a few of them exhibit substantial affinity, with K_i values in the low nanomolar concentrations. The most active among the twelve was the *para*-iodo substituent compound (**16**), which has a greater binding affinity than the nonnucleoside analog, dipyridamole. We present the synthesis and flow cytometry investigation of the SAR of these new halogenated *es* transporter inhibitors.

Structures and synthetic approach are illustrated in Scheme 1. Alkylation of commercially available 6-mercaptopurine riboside (**3**) with substituted benzyl halides (**4**) in dimethylformamide (DMF), in the presence of potassium carbonate, gave *S*-benzylated derivatives of 6-mercaptopurine riboside (**5**–**16**).[12] The structures and purities of the compounds were confirmed using NMR, mass spectroscopy, and elemental analysis.

Compounds synthesized for the study are as shown below.

Compound	X (Halogen substituent position)
5	2-Bromo
6	3-Bromo
7	4-Bromo
8	2-Chloro
9	3-Chloro
10	4-Chloro
11	2-Fluoro
12	3-Fluoro
13	4-Fluoro
14	2-Iodo
15	3-Iodo
16	4-Iodo



Scheme 1. Reaction condition: (a) Potassium carbonate/ DMF.

Table 1. Flow cytometrically-determined K_i values

Compound	X (Halogen substituent position)	K_i (nM)
1		0.70
2		8.79
5	2-Bromo	134.23
6	3-Bromo	20.54
7	4-Bromo	9.98
8	2-Chloro	99.49
9	3-Chloro	15.96
10	4-Chloro	14.23
11	2-Fluoro	19.87
12	3-Fluoro	11.21
13	4-Fluoro	9.02
14	2-Iodo	16.13
15	3-Iodo	13.79
16	4-Iodo	3.88

A flow cytometric competitive binding assay was employed to study the *es* nucleoside transporter binding affinity of the synthesized compounds. The advantages of flow cytometry over conventional radioligand binding assays are that, it eliminates the hazard of radiation exposure and disposal problems associated with radioactive waste, as well as allows the assay to be performed using much fewer cells. With flow cytometry we can use as few as 5000 cells as compared to 2 million cells for comparable radioligand assay. The cell line used to carry out the assay was the human chronic myelogenous leukemia, K562 cell line. A fluorescent ligand, 5-(SAENTA)-X8-fluorescein, which has high affinity for the *es* transporter,[13] was used in competition with the synthesized compounds[14] to carry out the competitive binding assay.[15]

Within this new series of 6-*S*-(halogen substituted benzyl) thioinosine analogs, compounds **5**–**16** exhibited a wide range of binding affinities toward the *es* nucleoside transporter. The same was measured by their ability to displace the *es* nucleoside transporter specific ligand, shown by the K_i values in Table 1.

Table 1 shows the inhibitory constant values of the compounds ranging from a low nanomolar concentration for compounds **13** and **16** to high nanomolar concentrations for compounds **5** and **8** with at least, a 30-fold difference in binding affinity between the most active and the least active compounds. A common trend in binding affinity among the isomers in each set for all the halogen substituents is readily apparent, that is *para*>*meta*>*ortho*. This is consistent with the reported SAR for nitro substitution as well.[3] Of the new analogs, compound **16**, which has a *para*-iodo substituent, proved to be better than, the widely used nonnucleoside nucleoside transport inhibitor dipyridamole as the ligand of the *es* transporter. Another analog, compound **13**, which has a *para*-fluoro substituent, exhibited a binding affinity comparable to that of dipyridamole.

It has been recognized that electron-withdrawing substituents are preferred in the *para*-position for high affinity binding of NBMPR analogs at the *es* transporter.[3] However, the effect of hydrophobicity of the

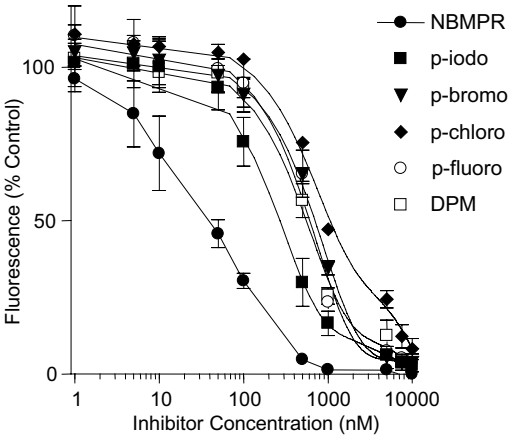


Figure 1. Equilibrium displacement of SAENTA-fluorescein ligand by new 6-*S*-(halogen substituted benzyl) thioinosine analogs of NBMPR in K562 cells. Cells were incubated with 30 nM SAENTA-fluorescein in the presence or absence of inhibitor for 45 min at room temperature and analyzed by flow cytometry (FACSCalibur®). Data was collected on 5000 cells per sample, and mean channel numbers were used as a measure of fluorescence output from ligands. DPM = dipyridamole.

analogs has not been well examined. The present results indicate that both increasing hydrophobicity and electron withdrawing capacity favor *es* transporter binding. The results obtained in the present study do support that notion. The optimum combination of these properties among the halogens in this regard appears to reside in the *para*-iodo compound (**16**). The enhanced affinity of the fluoro compound (**13**) relative to the bromo (**7**) and chloro (**10**) compounds stems possibly from the higher electron withdrawing ability of the fluorine substituent. This information coupled with information regarding the bioactive conformation of NBMPR that we are gathering from conformationally restricted analogs[16] should be useful in our efforts at modeling *es* nucleoside transporter inhibitors. The substantial binding affinity of some of these new halogen-substituted NBMPR analogs, especially compound **16** (K_i 3.88 nM), marks them as new potential leads for developing better *es* transporter inhibitors for therapeutic applications.

Acknowledgements

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14. To a solution of 6-mercaptopurine riboside (200 mg, 0.70 mmol) in DMF (2.5 mL) was added the corresponding halogen substituted benzyl halides (0.84 mmol) and potassium carbonate (1 g, 7.23 mmol). The mixture was stirred overnight at ambient temperature. The mixture was evaporated in vacuo at a temperature below 45 °C to remove the DMF. The residue was dissolved in water and extracted with three 75 mL portions of ethyl acetate. The combined organic layer was washed once with brine, dried over magnesium sulfate, and evaporated in vacuo. The crude product was purified using silica gel chromatography followed by recrystallization from methanol, to afford the pure compounds.
15. The *es* nucleoside transporter binding ability of the compounds was evaluated using a flow cytometric assay.[13] Human leukemia K562 cells, grown in RPMI 1640 medium were washed once and resuspended at 1.6×10^5 cells/mL in phosphate buffered saline at pH 7.4, and incubated with 5-(SAENTA)-X8-fluorescein (30 nM) in the presence or absence of varying concentrations of the test compounds at room temperature for 45 min. Flow cytometric measurements of cell associated fluorescence were then performed with a FACSCalibur (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, 5000 cells were analyzed from suspensions of 5×10^5 cells/mL. The units of fluorescence were arbitrary channel numbers. Percentage (%) of control (i.e., *es* transporter-specific fluorescence in the presence of SAENTA-fluorescein without test compounds) was calculated for each sample using Eq. 1.

$$\%\ \text{control} = (SF_s) \times 100/(SF_f) \quad (1)$$

where SF_s is the *es* transporter-specific fluorescence of test samples, and SF_f, is the *es* transporter-specific fluorescence of the SAENTA-fluorescein ligand standard in mean channel numbers. The results obtained were entered in the PRISM program (GraphPad, San Diego, CA) to derive the concentration dependent curves (examples are shown in Fig. 1). From these curves, the IC_{50} values were computed and used to calculate inhibition constants (K_i) values from Eq. 2.

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

where, [L] and K_L are the concentration and the K_d value of SAENTA-fluorescein, respectively. The K_i values were used to compare the abilities of the new compounds to displace the *es* transporter-specific ligand 5-(SAENTA)-X8-fluorescein, and for that matter their affinity for the *es* transporter.

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