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## Inhibition of Nucleoside Transport by p38 MAPK Inhibitors\*

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**While investigating the ability of p38 MAPK to regulate cytarabine (Ara C)-dependent differentiation of erythroleukemia K562 cells, we observed effects that indicated that the imidazoline class of p38 MAPK inhibitors prevented nucleoside transport. Incubation of K562 cells with SB203580, SB203580-iodo, or SB202474, an analogue of SB203580 that does not inhibit p38 MAPK activity, inhibited the uptake of [<sup>3</sup>H]Ara C or [<sup>3</sup>H]uridine and the differentiation of K562 cells. Consistent with the effects of these compounds on the nitrobenzylthioinosine (NBMPR)-sensitive equilibrative nucleoside transporter (ENT1), incubation with SB203580 or SB203580-iodo eliminated the binding of [<sup>3</sup>H]NBMPR to K562 cells or membranes isolated from human erythrocytes. Furthermore, using a uridine-dependent cell type (G9c), we observed that SB203580 or SB203580-iodo efficiently inhibited the salvage synthesis of pyrimidine nucleotides *in vivo*. Thus these studies demonstrate that the NBMPR-sensitive equilibrative nucleoside transporters are novel and unexpected targets for the p38 MAPK inhibitors at concentrations typically used to inhibit protein kinases.**

Nucleoside transporters are essential for the salvage pathway synthesis of nucleic acids and the transport of a wide range of nucleoside analogues used in the treatment of human neoplastic and viral diseases, including leukemia and AIDS (1, 2). Most mammalian cells co-express several nucleoside transporter isoforms at the plasma membrane that differ in their cation-dependent, permeant selectivities and inhibitor sensitivities and can generally be assigned to one of two major classes designated as the equilibrative or concentrative transporters (3). The Na<sup>+</sup>-independent equilibrative nucleoside

transporters (ENTs)<sup>1</sup> transport nucleosides by chemical gradients and can be further divided into equilibrative-sensitive (es) and equilibrative-insensitive (ei) (encoded by ENT1 and ENT2 gene, respectively) based on sensitivity, or insensitivity, to the high affinity antagonist nitrobenzylthioinosine (NBMPR) (3, 4). The es transporters are widely expressed, whereas ei transporters are found as a minor component in intestine, hematopoietic cells, skeletal muscle, and cardiovascular tissue (3–5).

The MAPKs are a large family of enzymes responsible for relaying cell surface signals to the nucleus and other intracellular targets (6). Not surprisingly these enzymes are highly desirable targets for pharmacological inhibitors of cell signaling. SB203580 was identified as one of the first highly selective inhibitors of the stress-activated p38 mitogen-activated protein kinases (p38 MAPKs) and was shown to block the production of tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$  release from lipopolysaccharide-stimulated monocytes (7). Considerable evidence now suggests that SB203580 exerts its anti-inflammatory actions by binding to the ATP binding site and inhibiting the activity of p38 MAPK (7–9). SB203580 and related analogues have also been shown to inhibit the production of interferon- $\gamma$ , interleukin-2, and tumor necrosis factor- $\alpha$  by lymphocytes stimulated with lipopolysaccharide, phorbol myristate acetate, sorbitol, and anti-CD3 plus anti-CD28 monoclonal antibodies (10–13). Since this initial discovery a wealth of studies has contributed to the discovery and design of a series of pyrimidine analogues as additional p38 MAPK inhibitors, some of which are currently in clinical trials for rheumatoid arthritis (14–16).

While investigating the involvement of p38 MAPK in the regulation of erythroid cell differentiation, we observed effects of SB203580 that were inconsistent with inhibition of this kinase. Instead our results suggested that there were additional effects on the transport of nucleosides or nucleoside analogues into K562 cells. In this report we describe evidence for the equilibrative nucleoside transporters as additional targets for inhibition by the imidazoline class of p38 MAPK inhibitors.

### MATERIALS AND METHODS

**Cell Cultures and Reagents**—Human erythroleukemia K562 cells and G9c cells were cultured as described earlier (17). Uridine (35–50 Ci/mmol) was purchased from ICN Biomedicals (Costa Mesa, CA). [<sup>3</sup>H]NBMPR (22.5 Ci/mmol) and [5-<sup>3</sup>H]cytosine- $\beta$ -D-arabinofuranoside ([5-<sup>3</sup>H]Ara C, 15–30 Ci/mmol) were from Moravsek Biochemicals (Brea, CA). Uridine, propidium, NBMPR, and Histopaque were obtained from Sigma. SB202190, SB202474, SB203580, SB203580-iodo, and SB220025 were purchased from Calbiochem-Novabiochem.

**Measurement of Erythroid Differentiation of K562 Cells by Benzidine Staining**—Erythroid differentiation was determined by measuring hemoglobin production by benzidine staining (17). Benzidine dihydrochloride (2 mg/ml) was prepared in 0.5 M (3%) acetic acid, and H<sub>2</sub>O<sub>2</sub> (1%)

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<sup>1</sup> The abbreviations used are: ENT, equilibrative nucleoside transporter; Ara C, cytarabine (1- $\beta$ -D-arabinofuranosylcytosine); NBMPR, nitrobenzylmercaptapurine ribonucleoside (6-[[4-(nitrobenzyl)thio]-9- $\beta$ -D-ribofuranosyl purine]; es, equilibrium-sensitive; ei, equilibrium-insensitive; MAPK, mitogen-activated protein kinase; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole; SB203580-iodo, 4-(3-iodophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; SB220025, 5-(2-amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinyl)imidazole; CHAPS, 3-[[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; SB202474, 4-(ethyl)-2-(4-methoxyphenyl)-5-(4-pyridyl)-1H-imidazole.

was added immediately before use. The cell suspensions were mixed with the benzidine solution in a 1:1 ratio and counted in a hemocytometer after 5 min. Blue cells were considered positive for hemoglobin, and at least 1000 cells were counted per sample.

**Uptake Assays of [<sup>3</sup>H]Ara C by K562 Cells**—Uptake assays of [<sup>3</sup>H]Ara C was conducted in RPMI 1640 medium at 37 °C. 5 × 10<sup>5</sup> K562 cells/sample were washed once with RPMI 1640 medium and then resuspended in 400 μl of RPMI 1640 medium. After preincubation with SB analogues or control Me<sub>2</sub>SO for 15 min, an equal volume of RPMI 1640 medium containing <sup>3</sup>H-labeled Ara C (100 nM) plus inhibitors or control Me<sub>2</sub>SO was added for 30 min. Uptake of <sup>3</sup>H-labeled Ara C was stopped by five rapid washes with ice-cold RPMI 1640 medium containing 200 μM unlabeled competing Ara C. Nonspecific binding was measured in the presence of 200 μM unlabeled Ara C. The cell pellets were lysed in 10% SDS before quantification of radioactivity.

**Uptake Assays of [<sup>3</sup>H]Uridine**—Uridine uptake assays were conducted as described previously (18) at room temperature in sodium-containing buffer (20 mM Tris/HCl, 3 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 2 mM CaCl<sub>2</sub>, 5 mM glucose, and 130 mM NaCl, pH 7.4) or sodium-free transport buffer (20 mM Tris/HCl, 3 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 2 mM CaCl<sub>2</sub>, 5 mM glucose, and 130 mM *N*-methyl-D-glucamine/HCl, pH 7.4). 5 × 10<sup>5</sup> K562 cells/sample or G9c cells were washed once with transport buffer and then resuspended in 400 μl of transport buffer. After preincubation with SB analogues, NBMPR, or Me<sub>2</sub>SO for 15 min, uptake assays were started by adding an equal volume of transport buffer containing <sup>3</sup>H-labeled uridine (10 μM) plus inhibitors or Me<sub>2</sub>SO. Uptake assays were stopped by five rapid washes with ice-cold transport buffer containing 1 mM unlabeled competing uridine. The cell pellets were lysed in 10% SDS before quantification of radioactivity.

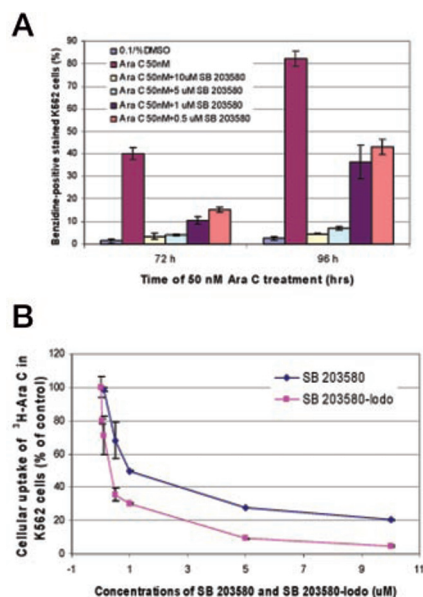
**Equilibrium Binding of [<sup>3</sup>H]NBMPR by Intact Cells**—Binding of NBMPR to K562 cells was measured using an assay described in detail previously (19). Briefly, total binding (5 × 10<sup>5</sup> cells/assay) was assessed in the transport buffer described above to which had been added graded concentrations (0.05–5 nM) of [<sup>3</sup>H]NBMPR. Alternatively, K562 cells were first incubated with various concentrations of SB analogues, and then 0.5 nM [<sup>3</sup>H]NBMPR was added. Binding was assessed at room temperature for 45 min. Nonspecific binding was determined by addition of 10 μM non-radioactive NBMPR in a set of replicate assay mixtures.

**Preparation of Membranes and Photoaffinity Labeling of Membranes with [<sup>3</sup>H]NBMPR**—Buffy coats from normal donors were obtained from the American Red Cross (Charlotte, NC). The human erythrocytes were isolated by density-gradient centrifugation (2600 rpm) using Histopaque 1077 (Sigma). Human erythrocyte ghost membranes were prepared as described earlier (20). [<sup>3</sup>H]NBMPR binding assays were performed at room temperature in 10 mM Tris (pH 7.1) containing 0.01% CHAPS (w/v). Incubations were initiated by adding an aliquot of 3 μg of erythrocyte ghost membranes to a glass tube containing the 0.5 nM [<sup>3</sup>H]NBMPR in the absence or presence of various concentrations of SB203580-iodo. Incubations were terminated after 45 min by dilution with 5 ml of ice-cold 10 mM Tris (pH 7.1) followed by rapid filtration through Whatman GF/B filters, which were then washed once with 5 ml of ice-cold 10 mM Tris (pH 7.1). Nonspecific binding of [<sup>3</sup>H]NBMPR was determined in the presence of 10 μM NBMPR.

**Analysis of Intracellular Nucleotides by High Performance Liquid Chromatography**—G9c cells (1 × 10<sup>7</sup>), treated as described in the figure legends, were harvested, and the samples were prepared and analyzed for nucleotides as described previously (21).

## RESULTS

**SB203580 Prevents the Ara C-dependent Differentiation of K562 Cells through Inhibition of Ara C Uptake**—Incubation of human K562 erythroleukemia cells with 50 nM Ara C increased the benzidine-positive staining of these cells in a time-dependent manner with ~80% differentiation occurring after 96 h. To determine whether p38 MAPK was involved in regulating the differentiation of these cells, K562 cells were co-incubated with Ara C and SB203580. Addition of 10 μM SB203580 inhibited greater than 90% of the Ara C-dependent differentiation of these cells after 96 h (Fig. 1A). To further investigate the influence of p38 MAPK inhibitors on this process, we examined whether these compounds affected the uptake of Ara C into cells. K562 cells were incubated with 50 nM [<sup>3</sup>H]Ara C and increasing concentrations of SB203580 or SB203580-iodo, an analogue of SB203580 with similar inhibitory effects on p38



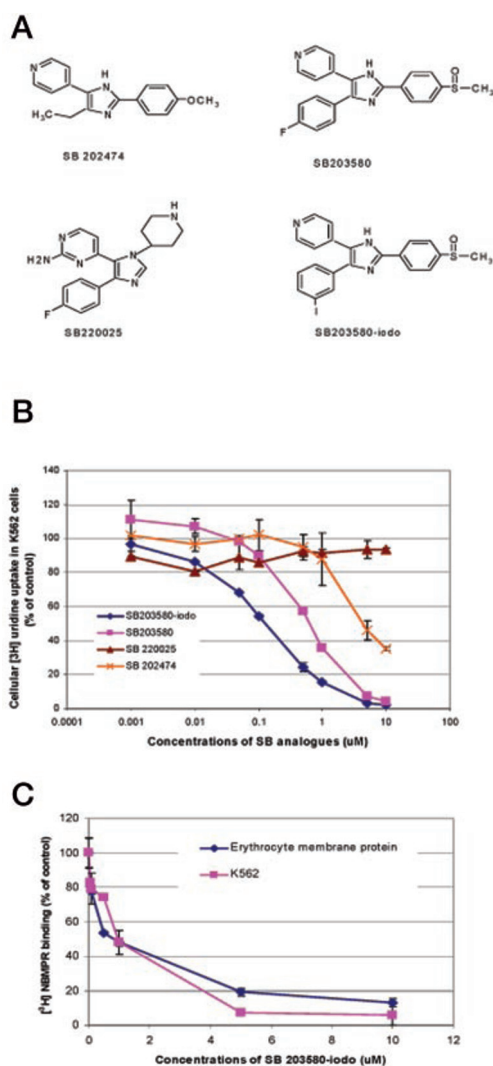
**FIG. 1. Effects of SB203580 on Ara C-induced erythroid differentiation of K562 cells and [<sup>3</sup>H]Ara C uptake in K562 cells.** A, K562 cells were exposed to 50 nM Ara C in the presence or absence of SB203580 at concentrations and times indicated. The percentages of hemoglobin-containing cells that stained positive for benzidine were obtained by counting at least 1000 cells/sample under microscopy using ×100 magnification. Data represent the mean ± S.D. of triplicate samples of *n* = 3 experiments. B, K562 cells were incubated with 50 nM [<sup>3</sup>H]Ara C in the presence of 0.1–10 μM of the p38 inhibitor SB203580, SB203580-iodo, or Me<sub>2</sub>SO (DMSO) for 30 min. The effects of SB203580 and SB203580-iodo on [<sup>3</sup>H]Ara C uptake were determined as described under “Materials and Methods,” and the data represent the assay of duplicate samples.

MAPK (22). Surprisingly, both SB203580 and SB203580-iodo significantly inhibited the uptake of [<sup>3</sup>H]Ara C into K562 cells in a dose-dependent manner (Fig. 1B).

**Inhibition of [<sup>3</sup>H]Uridine Uptake by p38 MAPK Inhibitors**—Because these results suggested that nucleoside transport was affected by these compounds, we examined whether the uptake of [<sup>3</sup>H]uridine was prevented by the p38 MAPK inhibitors. Incubation of K562 cells with four different SB derivatives (Fig. 2A) demonstrated that SB203580-iodo, SB203580, and SB202474 inhibited the uptake of [<sup>3</sup>H]uridine in a dose-dependent manner, whereas SB220025 was without effect (Fig. 2B). SB202474 does not inhibit p38 MAPK, whereas SB220025 inhibits this kinase with a *K<sub>i</sub>* similar to SB203580 (7, 14). Thus these results demonstrated that the effects on [<sup>3</sup>H]uridine uptake occurred independently of p38 MAPK inhibition. Analysis of the data revealed an IC<sub>50</sub> of 93.5 ± 1.5 and 689.4 ± 222.2 nM for SB203580-iodo and SB203580, respectively (Fig. 2B). In addition, the related compound SB202190 also inhibited [<sup>3</sup>H]uridine uptake in a dose-dependent manner (data not shown).

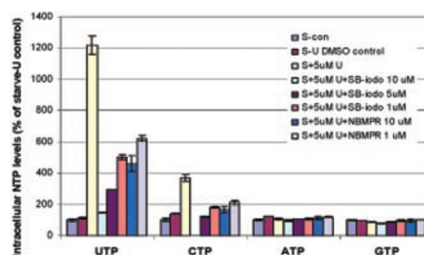
**Effects of p38 MAPK Inhibitors on Equilibrative Binding of [<sup>3</sup>H]NBMPR to Intact K562 Cells or Human Erythrocyte Membranes**—Since previous studies suggested that the majority of nucleoside uptake in K562 cells occurred by NBMPR-sensitive, equilibrative transporters (23), we investigated whether the uptake of [<sup>3</sup>H]NBMPR was prevented by the p38 MAPK inhibitors. Addition of [<sup>3</sup>H]NBMPR to K562 cells demonstrated that this compound was rapidly transported into K562 cells in a time-dependent manner (data not shown). Incubation of K562 cells with SB203580-iodo potently inhibited the uptake of [<sup>3</sup>H]NBMPR into these cells (Fig. 2C). Moreover, using isolated human erythrocyte membranes, we found that SB203580-iodo directly interfered with the binding of <sup>3</sup>H-labeled NBMPR to nucleoside transporter with an IC<sub>50</sub> of 0.5 μM (Fig. 2C).





**FIG. 2. Structures of SB class of p38 MAPK inhibitors and effects on [<sup>3</sup>H]uridine and [<sup>3</sup>H]NBMPR uptake in K562 cells and human erythrocyte membrane proteins.** *A*, the chemical structures of the four SB analogues used in this study are shown. *B*, the indicated compounds were tested for their capacity to inhibit the uptake of 5 μM [<sup>3</sup>H]uridine in K562 cells. The IC<sub>50</sub> of SB203580 and SB203580-ido on uridine uptake was determined by incubation with 5 μM [<sup>3</sup>H]uridine in the presence of 0.01–10 μM SB203580, SB203580-ido, or Me<sub>2</sub>SO control for 1 min. *C*, 3 μg of human erythrocyte membrane proteins or 5 × 10<sup>5</sup> K562 cells/sample were incubated in sodium-containing transport buffer containing 0.5 nM [<sup>3</sup>H]NBMPR in the presence of 0.05–10 μM of SB203580-ido. The effects of the SB compounds on binding of [<sup>3</sup>H]NBMPR were determined as described under “Materials and Methods.” Data are shown as percentage of control binding where the “control” was the binding of 0.5 nM [<sup>3</sup>H]NBMPR in the absence of inhibitors. Each point represents the mean ± S.D. from *n* = 2 experiments conducted in duplicate.

**Inhibition of Salvage Pyrimidine Nucleotide Synthesis by SB203580-ido or NBMPR-mediated Inhibition of Nucleoside Transport**—Finally, the efficacy of the SB derivatives in preventing the uptake and salvage of pyrimidine nucleosides was further evaluated using a uridine-dependent cell model system (G9c). The G9c cell line is a Chinese hamster ovary cell line that lacks the capacity for *de novo* pyrimidine synthesis and requires exogenous uridine to synthesize pyrimidine ribo- (or deoxy-) nucleotides for cell growth (24). G9c cells were first deprived of uridine for 24 h and then incubated with increasing concentrations of SB203580-ido, NBMPR, or Me<sub>2</sub>SO for an additional 8 h in the presence or absence of 5 μM uridine. Removal of uridine from the growth medium resulted in the



**FIG. 3. Effects of SB analogues and NBMPR on intracellular ribonucleotide pools in uridine-starved G9c cells.** G9c cells were starved for uridine for 24 h and then incubated with SB203580-ido (SB-ido), NBMPR, or Me<sub>2</sub>SO (DMSO) at the concentration indicated in the absence or presence of 5 μM uridine for an additional 8 h. Intracellular UTP, CTP, GTP, and ATP were extracted and measured as described under “Materials and Methods.” The levels of intracellular ATP, GTP, CTP, and UTP of unstarved G9c cells are 29.8, 6.8, 5.3, and 18.7 nmol/1 × 10<sup>7</sup> G9c cells, respectively; the levels of intracellular ATP, GTP, CTP, and UTP of starved G9c cells are 38.6, 10.7, 0.7, and 0.6 nmol/1 × 10<sup>7</sup> G9c cells, respectively. The data shown are plotted as the percentage of the G9c starvation control and represent the mean of duplicate samples. *S*, starvation; *S-con*, starvation control.

depletion of the UTP and CTP pools, whereas the purines, ATP and GTP, were unaffected (Fig. 3). Addition of 5 μM uridine to the growth medium restored the intracellular pyrimidine nucleotide pools; however, addition of SB203580-ido and NBMPR prevented pool restoration in a dose-dependent manner (Fig. 3). By contrast, coinubation of uridine-starved G9c cells with SB203580-ido or NBMPR alone for an additional 8 h had no obvious effect on pyrimidine pools in comparison with the uridine-starved G9c cells (data not shown). These results indicated that inhibition of nucleoside transporters by the SB analogues was sufficient to inhibit the salvage of pyrimidine nucleosides *in vivo*.

## DISCUSSION

The salvage of pyrimidine nucleosides is critically dependent on facilitated transport into cells. In addition, cellular uptake of chemotherapeutic nucleoside analogues such as Ara C or gemcitabine (2',2'-difluorodeoxycytidine) occurs by equilibrative nucleoside transport (25–28). The results of the current study demonstrate that the uptake of nucleosides and nucleoside analogues is potently inhibited by the imidazoline class of p38 MAPK inhibitors. While our results do not eliminate a role for p38 MAPK in Ara C-mediated differentiation of K562 cells, these data show that the SB compounds inhibit nucleoside transport independently of p38 MAPK activity. Specifically, SB202474, an analogue of SB203580 that does not inhibit p38 MAPK (7), dose dependently inhibited nucleoside transport. Consistent with this observation, incubation of K562 cells with SB202474 also prevented the Ara C-dependent differentiation of these cells in a dose- and time-dependent manner (72 h: control, 4.4 ± 0.8%; 10 μM SB202474, 4.8 ± 0.4%; 50 μM SB202474, 9.0 ± 0.4%; 50 nM Ara C, 33.7 ± 3.0%; 50 nM Ara C plus 10 μM SB202474, 20.1 ± 2.1%; 50 nM Ara C plus 50 μM SB202474, 8.8 ± 3.0%. 96 h: control, 8.2 ± 1.6%; 10 μM SB202474, 9.1 ± 2.0%; 50 μM SB202474, 9.9 ± 1.9%; 50 nM Ara C, 72.9 ± 6.9%; 50 nM Ara C plus 10 μM SB202474, 50.8 ± 6.6%; 50 nM Ara C plus 50 μM SB202474, 9.4 ± 1.5%). The observation that SB202474 was less effective than either SB203580 or SB203580-ido suggests that the absence of a fluorobenzyl ring may reduce the efficiency of nucleoside transport inhibition (see Fig. 2A). By contrast, SB220025, a novel inhibitor of p38 MAPK with similar potency (IC<sub>50</sub> = 60 nM) (29) to SB203580 (IC<sub>50</sub> = 48 nM) (7, 14), failed to inhibit nucleoside transport in K562 cells. Thus these results show that some SB analogues inhibit nucleoside transport in a p38 MAPK-independent man-

ner and provide insight into the structure/activity relationship of these compounds.

The results of our studies demonstrate that the equilibrative transporters are targets for inhibition by this class of p38 MAPK inhibitors. In human erythroleukemia (K562) cells, ~80–90% of total nucleoside transport activity occurs by equilibrative, NBMPR-sensitive (es) transport, whereas the remainder occurs by an NBMPR-insensitive (ei) transport process (23). Using K562 cells as a model system, we observed that greater than 90% of the uridine transport was inhibited by the SB compounds. Moreover using K562 cells or membranes from erythrocytes we demonstrated a specific, competitive inhibition of NBMPR binding, strongly indicating that the SB analogues inhibited the activity of human ENT1.

The pattern of nucleoside transporter expression has been shown to vary according to the cell line or origin (1). Our data demonstrating that the SB analogues completely prevented the salvage of pyrimidine nucleosides and that SB203580-iodo was more effective than NBMPR at inhibiting uridine transport or the repletion of intracellular pyrimidine nucleotide pools in G9c cells suggests additional effects of these compounds on the ei or concentrative transporters. Measuring uridine uptake in the presence of NBMPR in these cells demonstrated that the SB compounds inhibited both ei and es nucleoside transporters (data not shown). Whether there are similar inhibitory effects of these compounds on other types of nucleoside transporters (*i.e.* concentrative) remains to be determined.

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