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Anticancer nucleobase analogues 6-mercaptopurine and 6-thioguanine are novel substrates for equilibrative nucleoside transporter 2

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Abstract

Various antimetabolites of nucleobase analogues, such as 6-mercaptopurine (6-MP), 6-thioguanine (6-TG) and 5-fluorouracil (5-FU), are used for cancer treatments. The first step in nucleobase analogue drug therapy is entry of these compounds into tumor cells. Equilibrative nucleoside transporter 2 (ENT2) was previously reported to have the dual ability of transporting both nucleosides and nucleobases. In the present study, we investigated whether or not these nucleobase analogues are transported *via* ENT2, using mouse ENT2-overexpressing Cos-7 cells. The hypoxanthine uptake mediated by ENT2 was significantly reduced by the addition of 6-MP and 6-TG, and the inhibition of the hypoxanthine uptake by the 6-thiopurines was competitive. Transfection of ENT2 cDNA into Cos-7 cells resulted in an increase in 6-MP uptake. The 6-MP uptake *via* ENT2 showed clear time- and substrate concentration-dependent profiles, and was inhibited by 6-TG in an inhibitor concentration-dependent fashion. On the other hand, uracil was not a substrate for ENT2, and 5-FU had no effect on the hypoxanthine uptake *via* ENT2. Therefore, we concluded that 6-MP and 6-TG, but not 5-FU, are transported mediated by the same recognition site on ENT2 with hypoxanthine.

Keywords: Nucleoside; Nucleobase; 6-Thiopurine; Transporter; Chemotherapy

1. Introduction

Various structural analogues of nucleobase, such as 6-mercaptopurine (6-MP), 6-thioguanine (6-TG) and 5-fluorouracil (5-FU), are cytotoxic and have found therapeutic use as antineoplastic agents (Spiegelman et al., 1980; Elgemeie, 2003). Once transported inside the cells, nucleobase analogue drugs interfere with DNA repair and replication. The efficient entry of these nucleobase analogue drugs into tumor cells, but not into normal cells, is thought to be necessary for rendering cytotoxic drug treatments more selective toward tumors while sparing the normal tissues. Therefore, an understanding of transport mechanisms of the nucleobase analogues is critical in the development of improved chemotherapeutic strategies.

Kinetic studies on nucleobase transport in a variety of mammalian cells have been vigorously performed. Mammalian cells simultaneously exhibit multiple nucleobase transport processes (Koning and Diallinas, 2000). However, no cDNA encoding a functional mammalian nucleobase transporter has been cloned. On the other hand, there are a few reports about nucleobase transport mediated by nucleoside transporters (NTs). Five mammalian NTs, which are expressed in the plasma membrane of cells, have been cloned, and they have been classified into two distinct unrelated families based on Na^+ dependence (Griffith and Jarvis, 1996; Cass et al., 1998, 1999; Kong et al., 2004). The concentrative NTs (CNTs) are Na^+-dependent, and three CNT subtypes (CNT1, pyrimidine-preferring; CNT2, purine-preferring; CNT3, broadly selective) have been characterized. The Na^+-independent equilibrative NTs (ENTs) have broad substrate selectivity for purine and pyrimidine nucleosides. They have been classified, according to their sensitivity to the inhibitor nitrobenzylmercaptopurine riboside (NBMPR), into sensitive (ENT1) and insensitive (ENT2) systems. Previously, it was reported that ENT2, but not ENT1 and all CNTs, transported not only nucleosides but also several nucleobases (Ritzel et al., 2001; Yao et al., 2002). Overall, ENT2 is the only transporter identified as nucleobase transport system at the molecular level. However, the involvement of ENT2 in the uptake of 6-MP, 6-TG and 5-FU remains unclear.

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In this study, therefore, we examined whether or not 6-MP, 6-TG and 5-FU are substrates for ENT2, using mouse ENT2-overexpressing Cos-7 cells (Cos-7/ENT2).

2. Materials and methods

2.1. Chemicals

Nucleobases, 6-TG and 5-FU were from Wako Pure Chemical Ind. (Osaka, Japan), and 6-MP and NBMPR were purchased from Sigma Chemical Co. (MO, USA). Radioactive nucleobases and uridine were obtained from American Radiolabeled Chemicals Inc. (MO, USA), and [^{14}C]6-MP was purchased from MORAVEK Biochem. Inc. (CA, USA). All other reagents were of commercial or analytical grade requiring no further purification.

2.2. Cell culture

Cos-7 cells were maintained in Dulbecco's modified Eagle's MEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (MP Biochemicals, LLC, CA, USA) at 37 °C under a humidified atmosphere of 5% CO_2 in air.

2.3. Cloning of ENT2 cDNA

An ENT2 clone was obtained by amplification of cDNA derived from mouse Ehrlich ascites carcinoma cells, using the PCR approach with exTaq (TaKaRa, Shiga, Japan). A 5′ primer 5′-TGTGTGGAGCACTGGTGGTCC-3′ and a 3' primer 5′-TGCAATAGGACTGCGGCAAC-3′ derived from the reported sequence of mouse ENT2 (accession number: AF183397) were used. The PCR product was subcloned into pGEM-T vector (Promega Co., WI, USA), and sequenced. The peptide encoded by cloned ENT2 was identical to that previously reported (Kiss et al., 2000). The ENT2 cDNA was then inserted into pCI-neo expression vector (Promega Co.) with *Eco*RI and *Sal*I (ToYoBo, Osaka, Japan).

2.4. Transfection

For transfection, the ENT2 cDNA inserted into pCI-neo expression vector was purified with a Wizard® plus SV Miniprep DNA purification system (Promega Co.). An appropriate quantity of the required plasmid (65.8 ng DNA/cm^2) was diluted with 100 μL of OPTI-MEM reduced serum medium (Invitrogen, CA, USA) containing TransFectin™ Lipid Reagent (Bio-Rad Laboratories Inc., CA, USA), followed by incubation at room temperature for 30 min. The DNA–liposome complexes were added to Cos-7 cells cultured up to 70% confluency, and after 48 h culture, the cells were used for the uptake assay. Cos-7 cells transfected with pCI-neo expression vector were used as mock cells (Cos-7/pCI-neo).

2.5. Uptake assay

Uptake experiments were performed by the modified method of Nagasawa et al. (2003). Briefly, the uptake reaction was initiated by adding ^{3}H-labeled uridine or nucleobases (1 μCi/mL), or ^{14}C-labeled hypoxanthine or 6-MP (0.1 μCi/mL) to the cells. In the case of uridine uptake, the cells were treated with 100 nM NBMPR to completely block the uptake mediated by ENT1 in Cos-7 cells. After an appropriate time interval, the reaction was terminated by the addition of ice-cold phosphate-based saline containing an excess concentration of each non-radioactive substrate. The intracellular concentrations of radioactive substrates were determined with a liquid scintillation counter, following the method of Nagasawa et al. (2003). Protein concentrations were measured by the method of Bradford (1976) with bovine serum albumin (Sigma Chemical Co.) as the standard.

2.6. Statistical analysis

Cellular uptake was usually determined as the cell/medium ratio, which was obtained by dividing the uptake amount by the substrate concentration in the transport medium. Recombinant ENT2-mediated uptake was calculated by subtracting the uptake by Cos-7/pCI-neo from that by Cos-7/ENT2. The Michaelis

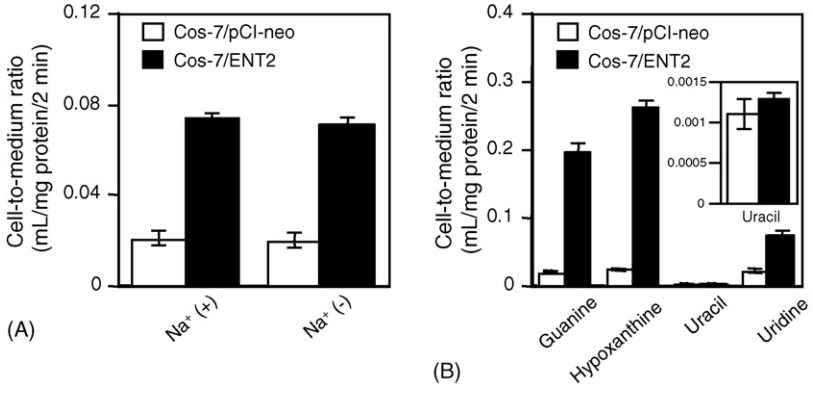

Fig. 1. Uptake of uridine and nucleobases by Cos-7/ENT2 and Cos-7/pCI-neo. (A) Cos-7/ENT2 and Cos-7/pCI-neo were incubated with uridine in HEPES–HBSS (pH 7.4) or choline-replaced HEPES–HBSS (pH 7.4) for 2 min at 37 °C. Each bar represents the mean ± S.E. ($n = 3$). (B) Cos-7/ENT2 and Cos-7/pCI-neo were incubated with a nucleobase or uridine in HEPES–HBSS (pH 7.4) for 2 min at 37 °C. The inset shows uracil uptake by Cos-7/ENT2 and Cos-7/pCI-neo. Each bar represents the mean ± S.E. ($n = 3$).

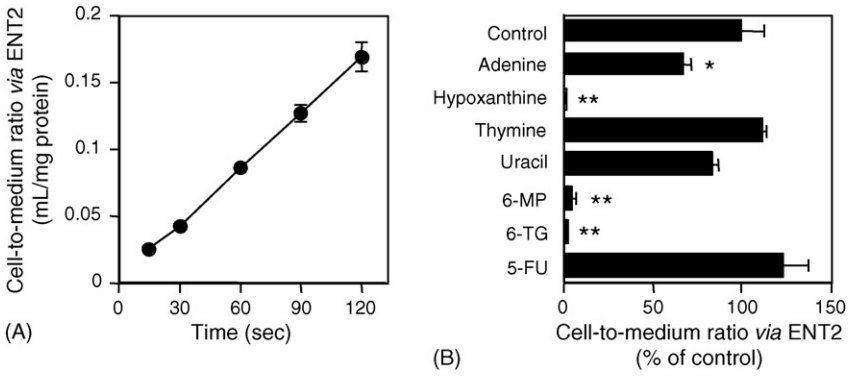

Fig. 2. Effects of nucleobases and their analogues on hypoxanthine uptake mediated by ENT2. (A) Cos-7/ENT2 and Cos-7/pCI-neo were incubated with hypoxanthine in HEPES–HBSS (pH 7.4) for the indicated times at 37 °C. Each point represents the mean ± S.E. ($n = 3$). (B) Cos-7/ENT2 and Cos-7/pCI-neo were incubated with hypoxanthine in HEPES–HBSS (pH 7.4) containing the indicated natural nucleobase (1 mM) or its analogue (100 μM) for 2 min at 37 °C. Each bar represents the mean ± S.E. ($n = 3$). $^{**}p < 0.01$, $^{*}p < 0.05$, significantly different from the control.

constant (K_m), maximal transport rate (V_{max}), and inhibition constant (K_i) were calculated on the basis of the Eadie–Hofstee equation. Comparison among groups was performed by means of analysis of variance (ANOVA, followed by Fischer's PLSD), differences with a p value of 0.05 or less being considered statistically significant.

3. Results

3.1. Uptake of uridine and nucleobases by Cos-7/ENT2 and Cos-7/pCI-neo

In order to confirm the functional expression of recombinant ENT2 in Cos-7/ENT2, the uptake of uridine, a typical substrate of NTs, by Cos-7/ENT2 and Cos-7/pCI-neo with or without extracelluar Na^+ was measured (Fig. 1A). The transfection of ENT2 cDNA into Cos-7 cells resulted in an increase in the uptake of uridine. The uridine uptake mediated by ENT2 was Na^+-independent, which was consistent with the transport characteristic of ENT2.

Since 6-MP, 6-TG and 5-FU are analogues of hypoxanthine, guanine and uracil, respectively, the uptake of these nucleobases by Cos-7/ENT2 and Cos-7/pCI-neo was measured. The uptake of guanine and hypoxanthine, but not uracil, by Cos-7/ENT2 was substantially greater than that by Cos-7/pCI-neo, and the ENT2-mediated uptake of these purine nucleobases was greater than that of uridine (Fig. 1B).

3.2. Effects of nucleobases and their analogues on hypoxanthine uptake mediated by ENT2

The hypoxanthine uptake *via* ENT2 increased linearly up to 2 min (Fig. 2A). Based on this result, subsequent hypoxanthine uptake experiments were carried out for 2 min. The experiments of Fig. 2B examined the ability of nucleobases and their analogues to inhibit the hypoxanthine uptake mediated by ENT2. The hypoxanthine uptake *via* ENT2 was significantly reduced by the addition of purine nucleobases, 6-MP or 6-TG, whereas no significant inhibition of the ENT2-mediated hypoxanthine uptake by pyrimidine nucleobases or 5-FU was observed.

3.3. Effects of 6-MP and 6-TG on hypoxanthine uptake mediated by ENT2

The inhibitor concentration–effect relationships for inhibition by 6-MP and 6-TG of ENT2-mediated hypoxanthine uptake were investigated. The hypoxanthine uptake mediated by ENT2 was sensitive to 6-MP or 6-TG in an inhibitor concentration-dependent fashion (Fig. 3A and B). As shown in Fig. 3C and D, the hypoxanthine uptake *via* ENT2 with or without 10 μM 6-MP or 6-TG showed a clear substrate concentration-dependent profile, and the resulting kinetic constants for the hypoxanthine uptake are presented in Table 1. The K_m values for hypoxanthine uptake *via* ENT2 with 6-MP and 6-TG were 1.43- and 2.54-fold higher, respectively, than those without the 6-thiopurines. The

Table 1
Kinetic constants for uptake of hypoxanthine and 6-MP mediated by ENT2

	K_m (μM)	V_{max} (nmol/mg protein/min)	V_{max}/K_m (mL/mg protein/min)
Hypoxanthine uptake			
6-MP (−)	3.86 ± 0.26	0.627 ± 0.149	0.162 ± 0.035
6-MP (+)	5.53 ± 0.54	0.505 ± 0.092	0.090 ± 0.011
6-TG (−)	3.74 ± 0.50	0.510 ± 0.115	0.144 ± 0.012
6-TG (+)	9.49 ± 1.66	0.293 ± 0.045	0.031 ± 0.002
6-MP uptake	14.21 ± 1.06	0.579 ± 0.048	0.041 ± 0.001

Kinetic constants were calculated by means of the Eadie–Hofstee equation using the data in Figs. 3C and D and 4B. Each value represents the mean ± S.E.M. ($n = 3$).

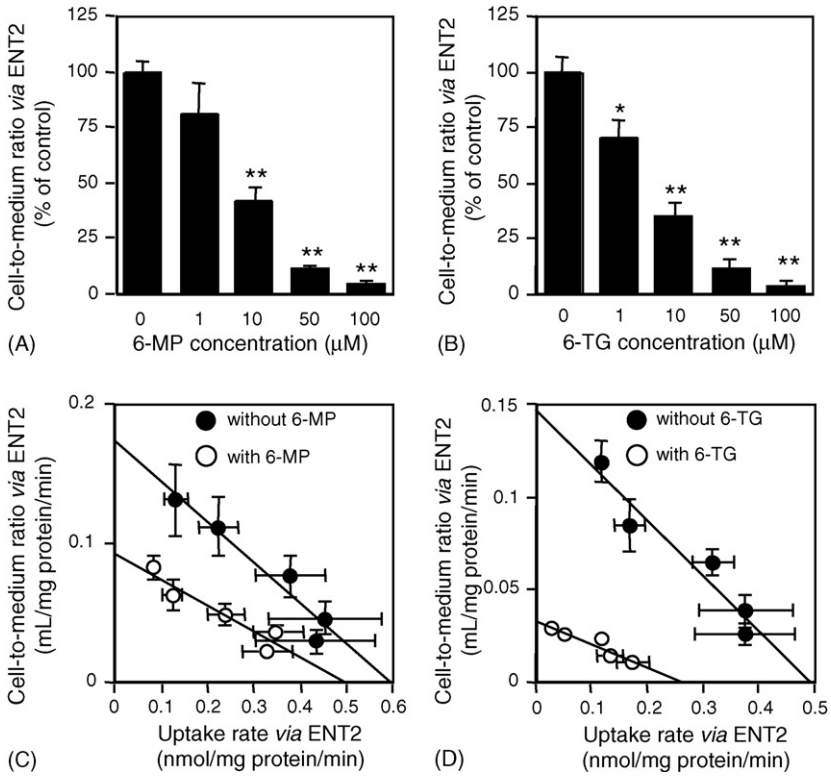

Fig. 3. Effects of 6-MP and 6-TG on the hypoxanthine uptake mediated by ENT2. (A and B) Cos-7/ENT2 and Cos-7/pCI-neo were incubated with hypoxanthine in HEPES–HBSS (pH 7.4) containing the indicated concentrations of 6-MP (A) or 6-TG (B) for 2 min at 37 °C. Each bar represents the mean ± S.E. ($n = 3$). $^{**}p < 0.01$, $^{*}p < 0.05$, significantly different from uptake without 6-thiopurine. (C and D) Cos-7/ENT2 and Cos-7/pCI-neo were incubated with 1–15 μM hypoxanthine in HEPES–HBSS (pH 7.4) in the presence or absence of 10 μM 6-MP (C) or 6-TG (D) for 2 min at 37 °C. Each point represents the mean ± S.E.M. ($n = 3$).

V_{max} value for the hypoxanthine uptake mediated by ENT2 was decreased in the presence of 6-TG to approximately 60%.

3.4. Transport characteristics of 6-MP mediated by ENT2

The time courses of 6-MP uptake by Cos-7/ENT2 and Cos-7/pCI-neo are shown in Fig. 4A. The 6-MP uptake by Cos-7/ENT2 was much greater than that by Cos-7/pCI-neo in a time-dependent fashion. As depicted in Fig. 4B, the ENT2-mediated uptake of 6-MP was saturated with a K_m value of 14.2 μM and a V_{max} value of 0.579 nmol/mg protein per min (Table 1). 6-TG inhibited the 6-MP uptake *via* ENT2 in an inhibitor concentration-dependent fashion (Fig. 4C).

4. Discussion

The permeation through the plasma membrane is a key step for the cytotoxicity of 6-MP, 6-TG and 5-FU, because their molecular targets are expressed intracellularly (Spiegelman et al., 1980; Elion, 1989). In the present study, we examined

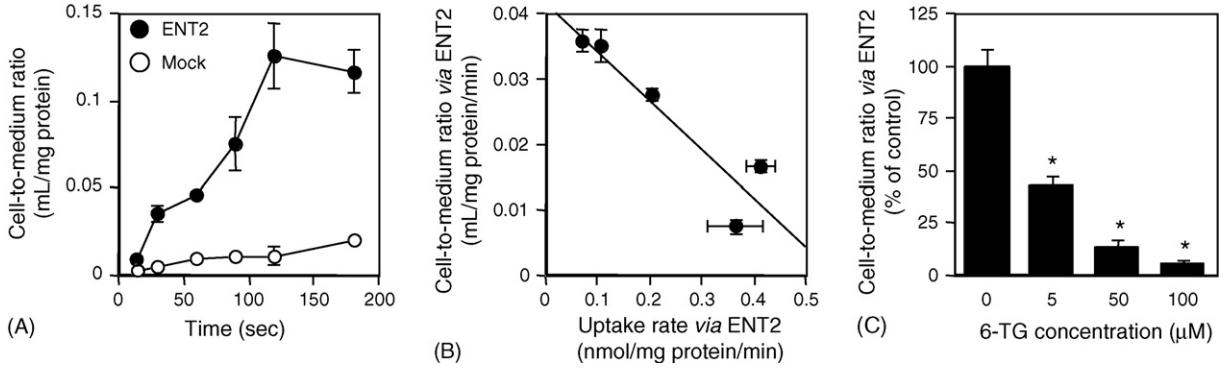

Fig. 4. Characteristics of 6-MP uptake mediated by ENT2. (A) Cos-7/ENT2 and Cos-7/pCI-neo were incubated with 6-MP in HEPES–HBSS (pH 7.4) for the indicated times at 37 °C. Each point represents the mean ± S.E. ($n = 3$). (B) Cos-7/ENT2 and Cos-7/pCI-neo were incubated with 2–50 μM 6-MP in HEPES–HBSS (pH 7.4) for 90 s at 37 °C. Each point represents the mean ± S.E.M. ($n = 3$). (C) Cos-7/ENT2 and Cos-7/pCI-neo were incubated with 6-MP in HEPES–HBSS (pH 7.4) containing the indicated concentrations of 6-TG for 90 s at 37 °C. Each bar represents the mean ± S.E. ($n = 3$). $^{*}p < 0.01$, significantly different from uptake without 6-TG.

whether or not 6-MP, 6-TG and 5-FU are substrates for ENT2, using ENT2-overexpressing Cos-7 cells.

6-MP was transported *via* ENT2 in time- and substrate concentration-dependent fashions. As shown in Figs. 1, 3 and 4, the uptake of hypoxanthine and 6-MP mediated by ENT2 was inhibited by 6-TG in an inhibitor concentration-dependent fashion. Furthermore, the ENT2-mediated uptake of guanine was three-fold greater than that of uridine. On the other hand, uracil was not a substrate for ENT2, and 5-FU had no effect on the hypoxanthine uptake *via* ENT2. Thus, the possibility of involvement of ENT2 in the uptake of 6-TG, but not 5-FU, was thought to be extremely high. The experiment presented in Table 1 demonstrated that the inhibition of ENT2-mediated hypoxanthine uptake by 6-MP and 6-TG was partially competitive, indicating that the 6-thiopurines share the same recognition site on the transporter with hypoxanthine.

Gu et al. (1995) have clearly explained that ENTs possess bidirectional activity. In order to understand the pharmacological roles of ENT2, it is important to clarify which direction of influx or efflux is predominant in cells. 6-MP and 6-TG are quickly converted into active metabolites intracellularly (Elion, 1989). The active metabolites are released *via* multidrug resistance protein families (Sampath et al., 2002), and then are metabolized by 5′-ecto-nucleotidase and purine nucleoside phosphorylase, cell-surface enzymes, into 6-MP and 6-TG (Silva et al., 2004; Eltzschig et al., 2006). These findings suggested that ENT2 plays a role in the uptake of 6-MP and 6-TG.

OAT1 and OAT3 have been identified as transport systems for 6-MP and 6-TG (Mori et al., 2004). However, these transporters have limited tissue distributions, such as in the kidney and brain (Mori et al., 2004; Wright and Dantzler, 2004), and there is no information on the expression of these transporters in cancer cells. Furthermore, the affinity for 6-MP uptake mediated by OAT1 and OAT3 was lower than that mediated by ENT2 (Mori et al., 2004). Therefore, these 6-thiopurines might be taken up into tumor cells *via* ENT2 rather than *via* OAT1 and OAT3.

6-MP and 6-TG have been mainly used for the treatment of leukemia (Elgemeie, 2003). The reasons were thought that the expression level of ENT2 in leukemia was approximately five-fold higher than that in a variety of tumor cells (Lu et al., 2002), and a relative reduction in the concentration of 6-MP and 6-TG in normal cells is likely to be caused by their efficient accumulation in leukemia. Furthermore, two 6-MP- or 6-TG-resistant T-lymphoblastic cells showed a substantial reduction in the expression level of mRNA for ENT2 (Fotoohi et al., 2006). Therefore, it was strongly implied that ENT2 is a factor determining the anti-tumor effect, but not the side effects, of 6-MP and 6-TG, in the case of therapy for ENT2-highexppresing tumors. Herein, the expression level of ENT2 in colon, ovarian and breast cancer cells was similar or higher compared to that in leukemia (Lu et al., 2002). Thus, there is the possibility that 6-MP and 6-TG are important agents for the induction and maintenance of remissions in patients not only with leukemia but also with colon, ovarian and breast cancers. Furthermore, ENT2 might become a molecular tool to explore biochemical modulators, which suppress side toxicity or enhance the anti-tumor effect of chemotherapeutic drugs. Namely, the combination of 6-thipurines with a specific activator for ENT2 might produce a synergistic anti-tumor effect against the aforementioned cancers. On the other hand, some protein-altering and splicing variants of ENT2, of which the transport characteristics differ from those of the wild-type, have been reported (Wu et al., 2005; Owen et al., 2006), suggesting that the tailor-made chemotherapy focused on ENT2 may be necessary for the appropriate usefulness of 6-MP and 6-TG.

The hypoxanthine uptake *via* ENT2 was significantly inhibited by 6-MP or 6-TG, and 6-TG ($K_i = 6.5$ μM) was a potent inhibitor for the hypoxanthine uptake by multiple mechanisms compared to 6-MP ($K_i = 23.3$ μM). As known, nucleobases and nucleosides are essential for cell survival and growth (Griffith and Jarvis, 1996). Tumor cells are thought to require more nucleobases and nucleosides than normal cells, because the expression of almost all NT isoforms has been described to increase in tumor cells compared to that in the respective normal cells (Goh et al., 1995; Micha et al., 2001). Thus, the inhibition of uptake of nucleobases and nucleosides *via* ENT2 by 6-MP or 6-TG has been demonstrated to be a newly discovered mechanism underlying their cytotoxicity, and furthermore, the modification of C(2) in the skeletal structure of a purine base might lead to the development of the most active antimetabolite of nucleobase analogue.

In summary, we demonstrated that 6-MP and 6-TG, but not 5-FU, are substrates for ENT2, and share the same recognition site on the transporter with hypoxanthine. This information will ultimately facilitate the efficient usefulness and development of antimetabolites of nucleobase analogues for cancer chemotherapy.

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