Molecular Pharmacology Fast Forward. Published on October 14, 2004 as doi:10.1124/mol.104.004408

MOLPHARM/2004/004408

Role of human nucleoside transporters in the cellular uptake of inhibitors of IMP dehydrogenase, tiazofurin and benzamide riboside

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Running title: Transport of nucleoside IMPDH inhibitors

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The number of text pages: 18

The number of tables: 4

The number of figures: 3

The number of references: 40

The number of words in the *Abstract*: 249

The number of words in the *Introduction*: 710

The number of words in the *Discussion*: 972

The abbreviations used are: IMPDH, inosine 5'-monophosphate dehydrogenase; IC_{50}, inhibitory concentration that reduces cell proliferation by 50%; NBMPR, nitrobenzylmercaptopurine riboside; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; h, human.

ABSTRACT

Benzamide riboside (BR) and tiazofurin (TR) are converted to analogs of NAD that inhibit IMP dehydrogenase (IMPDH), resulting in cellular depletion of GTP and dGTP and inhibition of proliferation. The current work was undertaken to identify the human nucleoside transporters involved in cellular uptake of BR and TR and to evaluate their role in cytotoxicity. Transportability was examined in *X. laevis* oocytes and *S. cerevisiae* that produced individual recombinant human nucleoside transporter types (hENT1, hENT2, hCNT1, hCNT2 or hCNT3). TR was a better permeant than BR with a rank order of transportability in oocytes of hCNT3 >> hENT1 > hENT2 > hCNT2 >> hCNT1. The concentration-dependence of inhibition of ^{3}H-uridine transport in *S. cerevisiae* by TR exhibited lower K_i values than BR: hCNT3 (5.4 *vs.* 226 μM), hENT2 (16 *vs.* 271 μM), hENT1 (57 *vs.* 168 μM) and hCNT1 (221 *vs.* 220 μM). In cytotoxicity experiments BR was more cytotoxic than TR to cells that were either nucleoside transport-defective or competent and transport-competent cells were more sensitive to both drugs. Exposure to nitrobenzylmercaptopurine ribonucleoside (NBMPR) conferred resistance to BR and TR cytotoxicity to hENT1-containing CEM cells, thereby demonstrating the importance of transport capacity for manifestation of cytoxicity. A breast cancer cell line with mutant p53 exhibited nine-fold higher sensitivity to BR than the otherwise similar cell line with wild-type p53, suggesting that cells with mutant p53 may be potential targets for IMPDH inhibitors. Further studies are warranted to determine if this finding can be generalized to other cell types.

INTRODUCTION

Inhibitors of inosine 5'-monophosphate dehydrogenase (IMPDH, EC 1.1.1.205) exhibit anticancer and antiviral activities (Franchetti and Grifantini, 1999). Benzamide riboside (1-β-D-ribofuranosylbenzene-3-carboxamide, BR) and tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide, TR) are C-nucleosides that exert their cytotoxic effects by forming analogs of NAD, wherein the nicotinamide moiety is replaced by the C-nucleoside base. Benzamide adenine dinucleotide (BAD) and thiazole-4-carboxamide adenine dinucleotide (TAD) inhibit IMP dehydrogenase (IMPDH) non-competitively, resulting in cellular depletion of guanylates (GTP and dGTP) and inhibition of the growth of several human tumor cell lines (Jayaram et al., 1982; Jayaram et al., 1993; Knight et al., 1987). Phase I/II clinical trials with TR in acute myelogenous leukemia patients showed significant reductions in leukemic cell burden (Jayaram et al., 1992; Tricot et al., 1989; Wright et al., 1996) and, as a result, TR is now approved as an orphan-drug by the U.S. Food and Drug Administration for treatment of chronic myelogenous leukemia in blast crisis (Grifantini, 2000).

In studies with cell lines, TR exhibited three-fold higher IC_{50} values (inhibitory concentration at which a 50% decrease in cell viability was observed) than BR (Gharehbaghi et al., 1994b). BR-induced cytotoxicity was attributed to induction of apoptosis and necrosis that was due to inhibition of IMPDH and, at higher concentrations, to induction of DNA double strand breaks, accompanied by reduction in pools of ATP (Grusch et al., 2002). BR, but not TR, inhibits malate dehydrogenase, suggesting different mechanisms of action (Gharehbaghi et al., 1994b) and involvement of the mitochondrial death pathway in induction of apoptosis (Khanna et al., 2004). The activity of IMPDH, the target enzyme responsible for cytotoxic action of BR and TR, appears to be linked to cancer transformation and progression, and its expression is

controlled by p53 in a reciprocal manner (Sherley, 1991; Stadler et al., 1994). Since mutation of p53 is a commonly observed step in oncogenesis (Soussi et al., 2000), inhibitors of IMPDH are candidate antitumor agents.

As C-nucleoside analogs, both TR and BR are likely to enter cells via nucleoside transporters. Earlier studies (Monks et al., 1985) demonstrated inhibition of uridine uptake by TR in erythrocytes and radiolabeled TR was transported into freshly isolated human erythrocytes with similar K_m and V_{max} values (170 μM and 55 nmol/10^6 cells/h, respectively) as uridine and thymidine (Monks et al., 1985), thus demonstrating that TR is a permeant for the transport process now known to be mediated by the human equilibrative transporter 1 (hENT1). Two families of nucleoside transporter proteins have been identified by molecular cloning and functional characteristics (Crawford et al., 1998; Griffiths et al., 1997; Ritzel et al., 2001; Ritzel et al., 1997; Ritzel et al., 1998). The human SLC 29 family of proteins contains four members (ENT) (Baldwin et al., 2004) of which two, hENT1 and hENT2, are known to transport nucleosides bidirectionally and exhibit broad permeant selectivities for nucleosides and in the case of hENT2 nucleobases (Yao et al., 2002). Three human members of the SLC28 family of sodium-dependent concentrative nucleoside transporters have been identified (Ritzel et al., 2001; Ritzel et al., 1997; Ritzel et al., 1998): hCNT1 (pyrimidine-nucleoside selective), hCNT2 (purine-nucleoside and uridine selective) and hCNT3 (broadly selective).

The current work examined the transportability of TR and BR by the human nucleoside transporter proteins to determine the role of various nucleoside transporters in uptake and cytotoxicity of BR and TR. Heterologous expression systems (*Xenopus laevis* oocytes and the yeast *Sachharomyces cerevisiae*) producing individual recombinant human nucleoside transporters were used to examine transportability by measuring inward fluxes of ^{3}H-labeled

drugs in the presence or absence of excess non-radioactive uridine. The relative affinities of the recombinant transporters for BR and TR were assessed by calculating K_i values from the concentration dependence of inhibition of ^{3}H-uridine transport in yeast using a high-throughput assay described previously (Zhang et al., 2003). Cytotoxicity studies were conducted in cultured human T-lymphoblastoid cells (CCRF-CEM and derivatives) in the presence or absence of inhibitors of equilibrative nucleoside transport to assess the importance of transportability of BR and TR in cytotoxicity. Cytotoxicity studies were also conducted in MN1 (wild-type p53) and MDD2 (mutant p53) breast adenocarcinoma cell lines to assess the role of p53 in manifestation of cytotoxicity to inhibitors of IMPDH. IMPDH expression is downregulated by p53 tumor suppressor gene (Liu et al., 1998; Sherley, 1991; Stadler et al., 1994).

Materials and Methods

Chemicals. [^{3}H]Benzamide riboside (specific radioactivity 175 mCi/mmol) was custom synthesized by American Radiolabeled Chemicals, St. Louis, MO, and [5-^{3}H]tiazofurin was obtained from Research Triangle Institute, Research Triangle Park, NC. NBMPR, dilazep, mineral oil, and unlabeled nucleosides were obtained from Sigma Chemical Company (Mississauga, Ontario). [5,6 ^{3}H]Uridine with a specific activity of 41.2 Ci/mmol was obtained from Moravek Biochemicals (Brea, CA) and its purity was >97% as confirmed by HPLC analysis. Tissue culture (96-well) plates, tissue culture medium Roswell Park Memorial Institute (RPMI), Buffalo, NY 1640 medium, Dulbecco's Minimal Eagle's Medium (DMEM), and fetal bovine serum were purchased from Gibco BRL (Burlington, Ontario). The CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit was obtained from Promega (Madison, WI). Ecolite was purchased from ICN Pharmaceuticals (Montreal, Quebec) and 550 silicone oil from BC Bearings (Edmonton, Alberta).

Production of recombinant transporters and measurement of radioisotope uptake in oocytes of *Xenopus laevis.* Production of recombinant human nucleoside transporters in oocytes was as described earlier (Ritzel et al., 2001). T3 polymerase, T7 polymerase, and SP6 polymerase were used to transcribe linearised plasmids, in the presence of the m7GpppG cap, using the MEGAscript transcription system (Ambion). Remaining template was removed by DNAase 1 digestion. Oocytes were then microinjected with either 20 nL of water alone (controls) or 20 nL of water containing 20 ng RNA transcripts (test samples). Unless otherwise indicated, uptake of ^{3}H-BR and ^{3}H-TR (20 μM) was measured three days post-injection. Experiments were performed at room temperature on groups of 10-12 oocytes in medium containing 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.5).

Following the incubation period, extracellular label was removed by six washes in ice-cold medium and individual oocytes were dissolved in 5% SDS (w/v) for quantitation of oocyte-associated ^{3}H by liquid scintillation counting (LS 6000 IC, Beckman).

Production of recombinant transporters and measurement of radioisotope uptake in *S. cerevisiae*. Construction of the *S. cerevisiae* expression systems for hENT1, hENT2, hCNT1, and hCNT3 was as described earlier (Visser et al., 2002; Zhang et al., 2003). Yeast harboring plasmids containing cDNAs that encode hENT1, hENT2, hCNT1 or hCNT3 were maintained in logarithmic growth phase in complete minimal medium with 2% glucose (CMM/GLU pH 7.4). The levels of production of hCNT2 in yeast were not sufficient to undertake experiments with either BR or TR. Transport experiments were conducted using a high-throughput assay described earlier (Zhang et al., 2003) that employed 96-well plates and a semi-automated cell harvester (Micro96^{TM} HARVESTER, Skatron instruments, Norway). The relative affinities of the transporters for BR and TR were assessed by measuring their abilities to inhibit the uptake of ^{3}H-uridine. Yeast producing recombinant hENT1, hENT2, hCNT1 or hCNT3 were incubated with graded concentrations of BR and TR in the presence of 1 μM ^{3}H-uridine for 20 and 10 min, respectively. Each experiment was repeated at least three times. Non-specifically associated radioactivity was determined in the presence of 10 mM non-radioactive uridine, and these values were subtracted from total uptake values. Data were subjected to nonlinear regression analysis using Graph Pad Prism Version 3.0 Software to obtain IC_{50} (inhibitory concentration 50%) values for BR and TR; K_i values were calculated using the Cheng and Prusoff equation (Cheng and Prusoff, 1973) and the K_m values for uridine (Visser et al., 2002; Zhang et al., 2003).

Growth and maintenance of cell lines. CCRF-CEM, originally obtained from Dr. W.T. Beck (formerly at St. Jude Children's Research Hospital, now at University of Illinois at

Chicago) and hereafter referred to as CEM, is a human T-lymphoblastoid cell line that exhibits endogenous hENT1-mediated nucleoside transport (Lang et al., 2001). CEM-ARAC-8C (hereafter referred to as CEM-ARAC) is a nucleoside transport-defective subline that was selected for resistance to cytarabine (Ullman et al., 1988). Stable transfectants of CEM-ARAC producing either hENT2, hCNT1 or hCNT2 were generated by electroporation and selection in geneticin as described earlier (Lang et al., 2001; Lang et al., 2004). hENT2 stable transfectants were produced by T. Lang (unpublished results) using procedures described previously (Lang et al., 2001; Lang et al., 2004). The cloned transfectants, CEM-ARAC/hENT2-TLET2, CEM-ARAC/hCNT1-TLCT1 and CEM-ARAC/hCNT2-D2, are hereafter referred to as CEM-ARAC/hENT2, CEM-ARAC/hCNT1 and CEM-ARAC/hCNT2, respectively. CEM, CEM-ARAC and the stably transfected CEM-ARAC cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (v/v) and the transfected cell lines were maintained in the presence of 0.25 μM tubercidin, 0.5 μM cytarabine and geneticin as described previously (Lang et al., 2001).

Dr. C. Dumontet (INSERM 453, France), generously provided the MN-1 (wild-type p53) and MDD-2 (mutant p53) derived from human breast carcinoma cell line MCF-7 (Galmarini et al., 2002) with permission from Dr. M. Oren (The Weizmann Institute of Science, Rehovat, Israel). The MDD-2 line was derived from MCF-7 by transfection with a plasmid containing dominant negative mutant p53 (which encodes a non-functional p53 protein), and the MN-1 line was generated from MCF-7 by transfection with an empty plasmid. Cells were maintained in Dulbecco's minimum essential medium containing 10% fetal bovine serum, 1% L-glutamine, 2% penicillin-streptomycin and neomycin (0.4 mg/ml). All cultures were kept at 37^{o}C in 5%

CO_2, 95% air and subcultured every two to three days to maintain exponential growth. Transport and cytotoxicity experiments were performed with actively proliferating cells.

Cytotoxicity assays. Cytotoxicity experiments were conducted with the CellTiter 96 proliferation assay kit (Promega Corp, Madison, WI). Cells were seeded in 96-well plates at densities of 10,000 (48-h exposures) or 50,000 cells (4-h exposures) per well for CEM, CEM-ARAC and stably transfected CEM-ARAC cells. MN1 and MDD2 cells were plated at 20,000 cells per well and exposed to drugs for 4 h. Cells were exposed to graded concentrations of BR (0-50 μM) or TR (0-500 μM) and treated with MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]) reagent for assessment of cytotoxicity. IC_{50} values were calculated from non-linear regression analysis of data plotted as percentages of control values against the logarithm of drug concentrations.

RESULTS

Inward fluxes of BR and TR by human equilibrative and concentrative nucleoside transporters expressed in *Xenopus* oocytes. The transportability of BR and TR (for structures, see Fig. 1) was examined in the *Xenopus* oocyte expression system by comparing uptake of 20 μM ^{3}H-BR and ^{3}H-TR by recombinant hENT1, hENT2, hCNT1, hCNT2 and hCNT3. Transport was measured in oocytes microinjected with water alone or with water containing transcripts encoding each of the nucleoside transporters. Mediated fluxes, defined as the difference in uptake values between RNA-injected and water-injected oocytes, are shown in Fig. 2. ^{3}H-Uridine was included as a control permeant. The mediated fluxes (pmol/oocyte/30 min) of TR were generally higher than those of BR: hENT1 (9 *vs.* 1.5), hENT2 (7 *vs.* 0.85), hCNT1 (0.9 *vs.* 0.7), hCNT2 (5 *vs.* 0.4) and hCNT3 (115 *vs.* 4.3). The highest accumulation of TR was seen in oocytes producing hCNT3 followed by hENT1, hENT2 and hCNT2. Fluxes of TR in hCNT1-producing oocytes were only slightly above background. Although BR was a weaker permeant than TR for all the transporters tested, mediated uptake of BR was observed in hCNT3-, hENT1-, hENT2- and hCNT1-producing oocytes.

Comparison of transportability of ^{3}H-TR and ^{3}H-BR by hENTs and hCNTs produced in *S. cerevisiae*. Time courses for influx of ^{3}H-uridine, ^{3}H-TR and ^{3}H-BR were measured in a uridine transport-defective strain of yeast (Vickers et al., 2000) that contained either pYPhENT1, pYPhENT2, pYPhCNT1 or pYPhCNT3. Experiments were not conducted with pYPhCNT2 because of consistently low levels of expression. To determine the initial rates of uridine uptake, fluxes of 1 μM ^{3}H-uridine were measured over time intervals that provided initial rates of uptake. Uridine uptake was linear for 40 min for hENT1-, hCNT1- and hENT2-producing yeast and for 12 min for hCNT3-producing yeast (Visser et al., 2002; Zhang et al.,

2003). Uptake of uridine in all four strains was abolished by addition of 10 mM unlabeled uridine to assay mixtures. Transportability of 1 μM ^{3}H-TR and ^{3}H-BR was assessed in the presence or absence of excess unlabeled 10 mM uridine. Mediated transport of 1 μM TR by hENT1 and hENT2 was higher than that of BR. Uptake of ^{3}H-uridine, ^{3}H-BR and ^{3}H-TR by hENT1 was blocked by the addition of 10 mM non-radioactive uridine (data not shown). TR exhibited higher initial rates of transport (pmol/mg protein/min) than BR in yeast producing hCNT3 (89 *vs.* 0.8), hENT1 (20 *vs.* 3), hENT2 (7.7 *vs.* 0.5) but not hCNT1 (0.06 *vs.* 0.2). Initial rates of transport of BR and TR by the human nucleoside transporters produced in yeast are summarized in Table 1.

Concentration-effect relationships for BR and TR for inhibition of ^{3}H-uridine transport by recombinant hENT1, hENT2, hCNT1 and hCNT3 in yeast.

The relative sensitivities of hENT1, hENT2, hCNT1 and hCNT3 mediated transport of uridine to inhibition by TR and BR were determined in concentration-effect experiments, which provided a measure of the affinity of TR and BR for the various transporters. Transport of 1 μM ^{3}H-uridine in the absence or presence of increasing concentrations of either BR or TR was tested for 10 min in hCNT3-producing yeast and for 20 min in hENT1-, hENT2- and hCNT1-producing yeast (Fig.3). The IC_{50} values obtained from the data of Fig. 3 and the kinetic constants for uridine reported earlier (Visser et al., 2002; Zhang et al., 2003) were used to calculate apparent K_i values (Table 2). TR inhibited hCNT3-, hENT1- and hENT2-mediated transport of uridine more potently than BR. BR was a weak inhibitor of uridine transport for each of the human nucleoside transporters tested.

Cytotoxicity of BR and TR to CEM cells with a single nucleoside transport process.

Cytotoxicity of BR and TR was tested against a panel of CEM cells that were either nucleoside

transport-defective or nucleoside transport-competent to determine if the presence of nucleoside transport activity was important for manifestation of cytotoxicity. CEM cells exhibit endogenous ENT1-mediated nucleoside transport and CEM-ARAC cells are nucleoside transport-defective. Stable transfectants of CEM-ARAC producing either hENT2, hCNT1 or hCNT2 were generated by electroporation and selection in geneticin as described earlier (Lang et al., 2001; Lang et al., 2004). The IC_{50} values generated by exposing cells to graded concentrations of either BR (0-50 μM) or TR (0-500 μM) are summarized in Table 3. In addition, modulation of cytotoxicity of BR and TR by an inhibitor of equilibrative processes was also examined in CEM cells to determine the extent to which hENT1 plays a role in cytotoxicity. Cells in 96-well plates were exposed to BR or TR in the presence or absence of 1 μM NBMPR for either four or 48 h. Cells were first incubated for one h with NBMPR to ensure complete blockade of the hENT1-mediated process prior to addition of graded concentrations of drugs. BR was more cytotoxic than TR during both exposure conditions. The protective effects of NBMPR were approximately four-fold for BR but much smaller for TR-mediated cytotoxicity (Table 3). These results demonstrated that NBMPR protected cells against both BR- and TR-mediated cytotoxicity during drug exposures of long duration. However, although NBMPR protected cells against BR-induced cytotoxicity during short exposures, its effect on TR cytotoxicity during short exposures could not be quantified because of the high IC_{50} values for TR (>500 μM) both in presence and absence of NBMPR. Additionally, at higher concentrations of BR and TR, diffusion processes may overcome the mediated processes. Cytotoxicity experiments with cells possessing hCNT3 were not undertaken due to unavailability of a stable hCNT3 transfectant.

Cytotoxicity of BR and TR to p53 wild-type and mutant breast adenocarcinoma cell lines.

Tumor suppressor gene p53 is involved in the regulation of IMPDH levels in cells. Since p53 mutation is the most commonly observed step in tumerogenesis (Soussi et al., 2000), inhibitors of IMPDH, such as BR and TR, may be useful as antitumor agents. To test the effects of BR and TR on cytotoxicity towards cells expressing wild-type or mutant p53, a pair of breast cancer cell lines (derived from MCF-7) that expressed either wild-type p53 (MN-1) or mutant p53 (MDD-2) were used in cytotoxicity experiments. MN-1 and MDD-2 cells were exposed to graded concentrations of BR and TR for four hours and cytotoxicity was assessed with the MTS reagent. Exposure to TR had no effect on either cell line at concentrations as high as 500 μM, whereas BR was about nine times more cytotoxic to cells with mutant p53 than to cells that contained wild-type p53 (Table 4).

DISCUSSION

BR and TR are C-nucleoside prodrugs, which, after cellular uptake, are metabolized to potent inhibitors of IMPDH (Gharehbaghi et al., 1994a; Jayaram et al., 1982), an enzyme that is required for *de novo* synthesis of guanine nucleotides. Both drugs are active in various pre-clinical model systems (Jayaram et al., 1992; Rauko et al., 2001; Tricot et al., 1989; Wright et al., 1996) and TR was approved by the U.S. Food and Drug Administration for the treatment of patients with chronic myelogenous leukemia in blast crisis (Grifantini, 2000). Although the intracellular metabolism of BR and TR has been documented (Jager et al., 2002; Jayaram et al., 1999), relatively little is known about their routes of membrane permeation. Five nucleoside transporters have been identified in various human cell types and are known to be important in the cellular uptake of a variety of anticancer nucleoside drugs (Baldwin et al., 2004; Damaraju et al., 2003). The objectives of the present study were to assess the transportability of BR and TR by the human nucleoside transporters and to determine the importance of mediated transport in cytotoxicity of BR and TR. Since both drugs are nucleoside analogs, they may be transported across plasma membranes by one or more of the nucleoside transporter proteins known to be present in the plasma membranes of human cells. Identification of the transporters involved in the uptake of BR and TR would help in the design of chemotherapy treatments, since the distribution of nucleoside transporter types differs among normal and neoplastic tissues in humans (Ritzel et al., 2001; Ritzel et al., 1997; Ritzel et al., 1998). We have used three model expression systems (*X. oocytes*, *S. cerevisiae* and cultured cell lines) to characterize BR and TR transportability and to define the role of the transporters to BR- and TR-induced cytotoxicity.

Each of the five human nucleoside transporter cDNAs was expressed individually in *Xenopus* oocytes and the transportability of ^{3}H-BR and ^{3}H-TR was studied. Although rates of

transport of ^{3}H-TR were highest in oocytes producing recombinant hCNT3, oocytes producing either hENT1, hENT2 or hCNT2 also exhibited transport of TR, although at lower rates. Mediated accumulation of BR was lower than that of TR in the oocyte-expression system, with transport observed in oocytes producing hCNT3, hENT1 and, to a much lesser extent, hENT2 and hCNT1.

The initial rates of uptake of ^{3}H-TR and ^{3}H-BR, and of uridine as a control permeant, were compared in the yeast-expression system. The uptake of TR and BR was mediated by nucleoside transporters since it was inhibited by excess non-radioactive uridine. The relative binding affinities of recombinant hENT1, hENT2, hCNT1 and hCNT3 for TR and BR were determined by examining their ability to inhibit ^{3}H-uridine transport in yeast producing the relevant recombinant transporter. The calculated IC_{50} and K_i values showed that hCNT3 exhibited the highest affinity for TR, followed by hENT2 and hENT1 (Table 2). In contrast, the transporters exhibited lower affinities for BR, which was a weak inhibitor of all of the transporters tested.

The cytotoxicities of BR and TR were assessed in a panel of lymphoblastoid CEM cell lines that produced the various nucleoside transporter types. BR was consistently more cytotoxic to the cell lines than TR. Transport activity was demonstrated to be important for manifestation of cytotoxicity of both drugs (see Table 3), although at the highest concentrations BR and TR were cytotoxic to all cell lines, probably reflecting cellular uptake by passive diffusion. Hematopoietic cells possess predominantly hENT1, and NBMPR, a potent hENT1 inhibitor (Visser et al., 2002), protects such cells from toxicity to cytotoxic nucleoside drugs (Cass et al., 1992). Protection experiments with NBMPR showed four-fold protection against cytotoxicity with BR during both short and long exposures (Table 3).

It has been shown that IMPDH is downstream of p53 (Liu et al., 1998; Sherley, 1991; Sherley et al., 1995). Cytotoxicity of BR and TR was tested in a pair of wild-type p53 and mutant p53 breast cancer cell lines to evaluate the role of p53, which has been previously shown to regulate levels of IMPDH in tumors (Jayaram et al., 1999). These studies showed that cells with wild-type p53 were nine-fold more resistant (Table 4) to BR than cells with the mutated p53, presumably because of p53-dependent down-regulation of expression of IMPDH, the target enzyme for BR. TR was not cytotoxic at concentrations as high as 500 μM in either cell line. These results suggested that BR (and possibly other IMPDH inhibitors) may have activity against cells with mutant p53. More detailed studies using additional cell types that differ in p53 status are needed to determine if the activity of IMPDH inhibitors in p53 mutant cells is a generalizable finding. Such studies may lead to the use of IMPDH inhibitors in treatment of tumors with mutated p53 that exhibit resistance to conventional anti-cancer nucleoside drugs like gemcitabine, cladribine and fludarabine (Galmarini et al., 2002; Galmarini et al., 2003; Pettitt et al., 1999).

In conclusion, both BR and TR were permeants for nucleoside transporters. The relative K_i values for BR and TR inhibition of ^{3}H-uridine transport in *S. cerevisiae* indicated that the transporters had higher affinities for TR than for BR. hCNT3 exhibited the highest affinity for TR followed by hENT2 and hENT1. BR was more cytotoxic than TR at both short and long exposure times and the cell lines with nucleoside transport activity were more sensitive to BR. Protection to BR and TR was demonstrated in cells exposed to NBMPR, an inhibitor of hENT1-mediated transport, thereby demonstrating the importance of transport activity for the cytotoxic action of both drugs. The present studies also demonstrated that a cell line with mutant p53 was more sensitive to BR than its counterpart with wild-type p53. Future research with other model

systems is needed to determine if p53 mutant cells are a potential pharmacologic target of inhibitors of IMPDH.

References

Baldwin SA, Beal PR, Yao SY, King AE, Cass CE and Young JD (2004) The equilibrative nucleoside transporter family, SLC29. *Pflugers Arch* **447**:735-43.

Cass CE, King KM, Montano JT and Janowska-Wieczorek A (1992) A comparison of the abilities of nitrobenzylthioinosine, dilazep, and dipyridamole to protect human hematopoietic cells from 7-deazaadenosine (tubercidin). *Cancer Res* **52**:5879-86.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-108.

Crawford CR, Patel DH, Naeve C and Belt JA (1998) Cloning of the human equilibrative, nitrobenzylmercaptopurine riboside (NBMPR)-insensitive nucleoside transporter ei by functional expression in a transport-deficient cell line. *J Biol Chem* **273**:5288-93.

Damaraju VL, Damaraju S, Young JD, Baldwin SA, Mackey J, Sawyer MB and Cass CE (2003) Nucleoside anticancer drugs: the role of nucleoside transporters in resistance to cancer chemotherapy. *Oncogene* **22**:7524-36.

Franchetti P and Grifantini M (1999) Nucleoside and non-nucleoside IMP dehydrogenase inhibitors as antitumor and antiviral agents. *Curr Med Chem* **6**:599-614.

Galmarini CM, Clarke ML, Falette N, Puisieux A, Mackey JR and Dumontet C (2002) Expression of a non-functional p53 affects the sensitivity of cancer cells to gemcitabine. *Int J Cancer* **97**:439-45.

Galmarini CM, Voorzanger N, Falette N, Jordheim L, Cros E, Puisieux A and Dumontet C (2003) Influence of p53 and p21(WAF1) expression on sensitivity of cancer cells to cladribine. *Biochem Pharmacol* **65**:121-9.

Gharehbaghi K, Paull KD, Kelley JA, Barchi JJ, Jr., Marquez VE, Cooney DA, Monks A, Scudiero D, Krohn K and Jayaram HN (1994a) Cytotoxicity and characterization of an active metabolite of benzamide riboside, a novel inhibitor of IMP dehydrogenase. *Int J Cancer* **56**:892-9.

Gharehbaghi K, Sreenath A, Hao Z, Paull KD, Szekeres T, Cooney DA, Krohn K and Jayaram HN (1994b) Comparison of biochemical parameters of benzamide riboside, a new inhibitor of IMP dehydrogenase, with tiazofurin and selenazofurin. *Biochem Pharmacol* **48**:1413-9.

Grifantini M (2000) Tiazofurine ICN Pharmaceuticals. *Curr Opin Investig Drugs* **1**:257-62.

Griffiths M, Beaumont N, Yao SY, Sundaram M, Boumah CE, Davies A, Kwong FY, Coe I, Cass CE, Young JD and Baldwin SA (1997) Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. *Nat Med* **3**:89-93.

Grusch M, Polgar D, Gfatter S, Leuhuber K, Huettenbrenner S, Leisser C, Fuhrmann G, Kassie F, Steinkellner H, Smid K, Peters GJ, Jayaram HN, Klepal W, Szekeres T, Knasmuller S and Krupitza G (2002) Maintenance of ATP favours apoptosis over necrosis triggered by benzamide riboside. *Cell Death Differ* **9**:169-78.

Jager W, Salamon A and Szekeres T (2002) Metabolism of the novel IMP dehydrogenase inhibitor benzamide riboside. *Curr Med Chem* **9**:781-6.

Jayaram HN, Cooney DA, Grusch M and Krupitza G (1999) Consequences of IMP dehydrogenase inhibition, and its relationship to cancer and apoptosis. *Curr Med Chem* **6**:561-74.

Jayaram HN, Dion RL, Glazer RI, Johns DG, Robins RK, Srivastava PC and Cooney DA (1982) Initial studies on the mechanism of action of a new oncolytic thiazole nucleoside, 2-beta-D-ribofuranosylthiazole-4-carboxamide (NSC 286193). *Biochem Pharmacol* **31**:2371-80.

Jayaram HN, Lapis E, Tricot G, Kneebone P, Paulik E, Zhen W, Engeler GP, Hoffman R and Weber G (1992) Clinical pharmacokinetic study of tiazofurin administered as a 1-hour infusion. *Int J Cancer* **51**:182-8.

Jayaram HN, Zhen W and Gharehbaghi K (1993) Biochemical consequences of resistance to tiazofurin in human myelogenous leukemic K562 cells. *Cancer Res* **53**:2344-8.

Khanna N, Jayaram HN and Singh N (2004) Benzamide riboside induced mitochondrial mediated apoptosis in human lung cancer H520 cells. *Life Sci* **75**:179-90.

Knight RD, Mangum J, Lucas DL, Cooney DA, Khan EC and Wright DG (1987) Inosine monophosphate dehydrogenase and myeloid cell maturation. *Blood* **69**:634-9.

Lang TT, Selner M, Young JD and Cass CE (2001) Acquisition of human concentrative nucleoside transporter 2 (hcnt2) activity by gene transfer confers sensitivity to fluoropyrimidine nucleosides in drug-resistant leukemia cells. *Mol Pharmacol* **60**:1143-52.

Lang TT, Young JD and Cass CE (2004) Interactions of nucleoside analogs, caffeine, and nicotine with human concentrative nucleoside transporters 1 and 2 stably produced in a transport-defective human cell line. *Mol Pharmacol* **65**:925-33.

Liu Y, Bohn SA and Sherley JL (1998) Inosine-5'-monophosphate dehydrogenase is a rate-determining factor for p53-dependent growth regulation. *Mol Biol Cell* **9**:15-28.

Monks A, Marquez VE, Mao DT and Cysyk RL (1985) Uptake of 2-beta-D-ribofuranosylthiazole-4-carboxamide (tiazofurin) and analogues by the facilitated transport mechanism of erythrocytes. *Cancer Lett* **28**:1-8.

Pettitt AR, Clarke AR, Cawley JC and Griffiths SD (1999) Purine analogues kill resting lymphocytes by p53-dependent and -independent mechanisms. *Br J Haematol* **105**:986-8.

Rauko P, Novotny L, Dovinova I, Hunakova L, Szekeres T and Jayaram HN (2001) Antitumor activity of benzamide riboside and its combination with cisplatin and staurosporine. *Eur J Pharm Sci* **12**:387-94.

Ritzel MW, Ng AM, Yao SY, Graham K, Loewen SK, Smith KM, Ritzel RG, Mowles DA, Carpenter P, Chen XZ, Karpinski E, Hyde RJ, Baldwin SA, Cass CE and Young JD (2001) Molecular identification and characterization of novel human and mouse concentrative Na+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). *J Biol Chem* **276**:2914-27.

Ritzel MW, Yao SY, Huang MY, Elliott JF, Cass CE and Young JD (1997) Molecular cloning and functional expression of cDNAs encoding a human Na+-nucleoside cotransporter (hCNT1). *Am J Physiol* **272**:C707-14.

Ritzel MW, Yao SY, Ng AM, Mackey JR, Cass CE and Young JD (1998) Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na+/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine. *Mol Membr Biol* **15**:203-11.

Sherley JL (1991) Guanine nucleotide biosynthesis is regulated by the cellular p53 concentration. *J Biol Chem* **266**:24815-28.

Sherley JL, Stadler PB and Johnson DR (1995) Expression of the wild-type p53 antioncogene induces guanine nucleotide-dependent stem cell division kinetics. *Proc Natl Acad Sci U S A* **92**:136-40.

Soussi T, Dehouche K and Beroud C (2000) p53 website and analysis of p53 gene mutations in human cancer: forging a link between epidemiology and carcinogenesis. *Hum Mutat* **15**:105-13.

Stadler PB, Pennacchi J and Sherley JL (1994) Inosine-5'-monophosphate dehydrogenase activity is maintained in immortalized murine cells growth-arrested by serum deprivation. *Adv Enzyme Regul* **34**:91-106.

Tricot GJ, Jayaram HN, Lapis E, Natsumeda Y, Nichols CR, Kneebone P, Heerema N, Weber G and Hoffman R (1989) Biochemically directed therapy of leukemia with tiazofurin, a selective blocker of inosine 5'-phosphate dehydrogenase activity. *Cancer Res* **49**:3696-701.

Ullman B, Coons T, Rockwell S and McCartan K (1988) Genetic analysis of 2',3'-dideoxycytidine incorporation into cultured human T lymphoblasts. *J Biol Chem* **263**:12391-6.

Vickers MF, Yao SY, Baldwin SA, Young JD and Cass CE (2000) Nucleoside transporter proteins of Saccharomyces cerevisiae. Demonstration of a transporter (FUI1) with high uridine selectivity in plasma membranes and a transporter (FUN26) with broad nucleoside selectivity in intracellular membranes. *J Biol Chem* **275**:25931-8.

Visser F, Vickers MF, Ng AM, Baldwin SA, Young JD and Cass CE (2002) Mutation of residue 33 of human equilibrative nucleoside transporters 1 and 2 alters sensitivity to inhibition of transport by dilazep and dipyridamole. *J Biol Chem* **277**:395-401.

Wright DG, Boosalis MS, Waraska K, Oshry LJ, Weintraub LR and Vosburgh E (1996) Tiazofurin effects on IMP-dehydrogenase activity and expression in the leukemia cells of patients with CML blast crisis. *Anticancer Res* **16**:3349-51.

Yao SY, Ng AM, Vickers MF, Sundaram M, Cass CE, Baldwin SA and Young JD (2002) Functional and molecular characterization of nucleobase transport by recombinant human and rat equilibrative nucleoside transporters 1 and 2. Chimeric constructs reveal a role for the ENT2 helix 5-6 region in nucleobase translocation. *J Biol Chem* **277**:24938-48.

Zhang J, Visser F, Vickers MF, Lang T, Robins MJ, Nielsen LP, Nowak I, Baldwin SA, Young JD and Cass CE (2003) Uridine binding motifs of human concentrative nucleoside transporters 1 and 3 produced in Saccharomyces cerevisiae. *Mol Pharmacol* **64**:1512-20.

FOOTNOTES:

This work was supported by Canadian Cancer Society and Terry Fox operating grants from the National Cancer Institute of Canada and by the Alberta Cancer Board.

FIGURE LEGENDS

Fig. 1. **Structures of BR and TR.**

Fig. 2. Transport of BR, TR and uridine by recombinant human equilibrative (ENT) and concentrative (CNT) nucleoside transporters produced in *Xenopus* oocytes. Oocytes were injected with either 20 nL of water alone or that contained 20 ng of RNA transcripts. Uptake of 20 μM ^{3}H-uridine (open bars), ^{3}H-BR (filled bars) and ^{3}H-TR (hatched bars) during 30-min exposures at 20^{0}C was determined after three days in NaCl-containing transport medium. Each value represents the mean of 10-12 oocytes. Mediated fluxes of TR, BR and uridine are defined as the difference in uptake values between RNA-injected and water-injected oocytes and were plotted as pmol/oocyte per 30 min. The fluxes (Mean ± SEM) for TR and BR are in the following order: hCNT3 (115 ± 15 *vs.* 4.3 ± 0.7); hENT1 (9 ± 0.3 *vs.* 1.5 ± 0.2); hENT2 (7 ± 0.4 *vs.* 0.85 ± 0.25); hCNT2 (5.2 ± 0.7 *vs.* 0.38 ± 0.24) and hCNT1 (0.93 ± 0.13 *vs.* 0.66 ± 0.18) respectively.

Fig. 3. Inhibition of recombinant hENT1-, hENT2-, hCNT1-, and hCNT3-mediated uptake of uridine by BR and TR. Yeast cells expressing pYPhENT1 (*open circles*), pYPhENT2 (*open triangles*), pYPhCNT1 (*open squares*) or pYPhCNT3 (*downward triangles*) were incubated for 20 min (10 min for hCNT3) in the presence of 1 μM ^{3}H-uridine with graded concentrations of BR (A) or TR (B). Uridine transport rates (mean ± SEM, n=4) in the presence of inhibitor are represented as percentages of the rates observed in the absence of inhibitor (*control*), and SEM values are not presented where the size of the point is larger than the SEM. Three separate experiments gave similar results. IC_{50} values were determined using GraphPad Prism version 3.0 software by nonlinear regression analysis. The K_i values are given in Table 2.

Table 1. Initial rates of uptake of BR and TR in *S. cerevisiae* producing human recombinant nucleoside transporters

Time courses of uptake of 1 μM ^{3}H-labeled uridine, TR or BR by hENT1, hENT2, hCNT1 or hCNT3-producing yeast were measured in the presence or absence of excess uridine as described in Materials and Methods. Initial rates (pmol/mg protein/min) ± S.E of uptake were calculated from the slopes of the linear regression lines using Graph Pad Prism 3.02 (Graph Pad Software, San Diego, CA); rates in the presence of 10 mM non-radioactive uridine were subtracted from rates in its absence to give rates of mediated uptake (i.e., transport).

Recombinant Transporter	Uridine	BR	TR
		pmol/mg protein/min	
hENT1	21.5 ± 1.1	3 ± 0.3	20 ± 0.7
hENT2	16.6 ± 1.02	0.5 ± 0.3	7.7 ± .2
hCNT1	7.9 ± 0.2	0.2 ± 0.05	0.06 ± .03
hCNT3	374 ± 19	0.8 ± .02	89 ± 4

Table 2. IC_{50} and K_i values for BR and TR inhibition of ^{3}H-uridine transport in yeast producing human nucleoside transporters

Rates of transport of 1 μM ^{3}H-uridine into yeast (fui 1::TRP) expressing pYphENT1, pYphENT2, pYphCNT1or pYphCNT3 were measured over 20 or 10 min in the presence of graded concentrations of BR and TR as described in Fig. 3. Mean IC_{50} ± S.E values were determined using Graph Pad prism, version 3.0, software, and then converted to K_i values by the Cheng and Prusoff (Cheng and Prusoff, 1973) equation using K_m values for uridine (Visser et al 2004) (Visser et al., 2002; Zhang et al., 2003).

Recombinant Transporter	BR		TR	
	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
hENT1	172 ± 21	168 ± 21	58 ± 5	57 ± 5
hENT2	272 ± 42	271 ± 42	16 ± 3	16 ± 3
hCNT1	224 ± 41	220 ± 40	225 ± 45	221 ± 44
hCNT3	252 ± 46	226 ± 41	6 ± 0.7	5.4 ± .6

Table 3. Chemosensitivities of CEM, CEM-ARAC/hENT2, CEM-ARAC/hCNT1, CEM-ARAC/hCNT2 and CEM-ARAC cells to BR and TR

Actively proliferating cells were exposed to graded concentrations of BR or TR in 96-well plates for 4 and 48 h as described in Materials and Methods. In protection experiments, CEM cells were treated with 1 μM NBMPR one hr prior to addition of BR or TR in the presence of 1 μM NBMPR. IC_{50} values were obtained from the MTS-cytotoxicity relationships and are means of at least two experiments, each conducted with six replicates. Mean ± S.E values were determined using Graph Pad prism, version 3.0, software.

	BR (IC_{50} μM)		**TR (IC_{50} μM)**	
	4 h	**48 h**	**4 h**	**48 h**
CEM*	11±2.9	1.7±0.28	>500	78±2.5
+ 1 μM NBMPR	46±5.7	6.0±0.75	>500	130±6.2
CEM-ARAC/hENT2	9±3.5	2.5±0.31	>500	110±5.1
CEM-ARAC/hCNT1	42±9.2	2.8±0.18	>500	130±21
CEM-ARAC/hCNT2	108±15	6.6±0.4	>500	106±15
CEM-ARAC**	107±17	6.5±0.42	>500	110±5

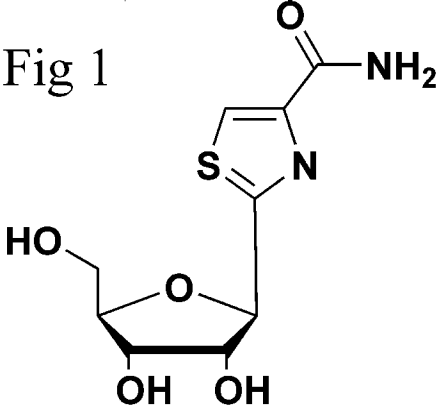
*has endogenous hENT1 activity.
** is nucleoside-transport defective.

Table 4. Chemosensitivity of MN-1 and MDD-2 cells exposed to graded concentrations of BR and TR

MN-1 (p53 positive) and MDD-2 (p53 negative) cells were exposed for four h to graded concentrations of BR (0-50 μM) and TR (0-500 μM) and assessed by the MTS assay as described in Materials and Methods. IC_{50} values obtained are means of three experiments; each conducted with six replicates. Mean ± S.E values were determined using Graph Pad prism, version 3.0, software.

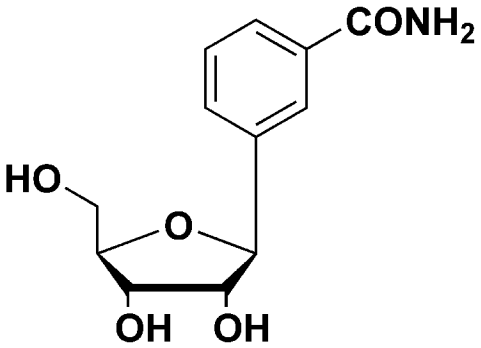
Drug	**MN-1 IC_{50} (μM)**	**MDD-2 IC_{50} (μM)**
BR	208±23	23±4.7
TR	>500	>500

Fig 1



Tiazofurin

2-β–D-Ribofuranosylthiazole-4-carboxamide



Benzamide Riboside

1-β–D-Ribofuranosylbenzene-3-carboxamide

Fig 2

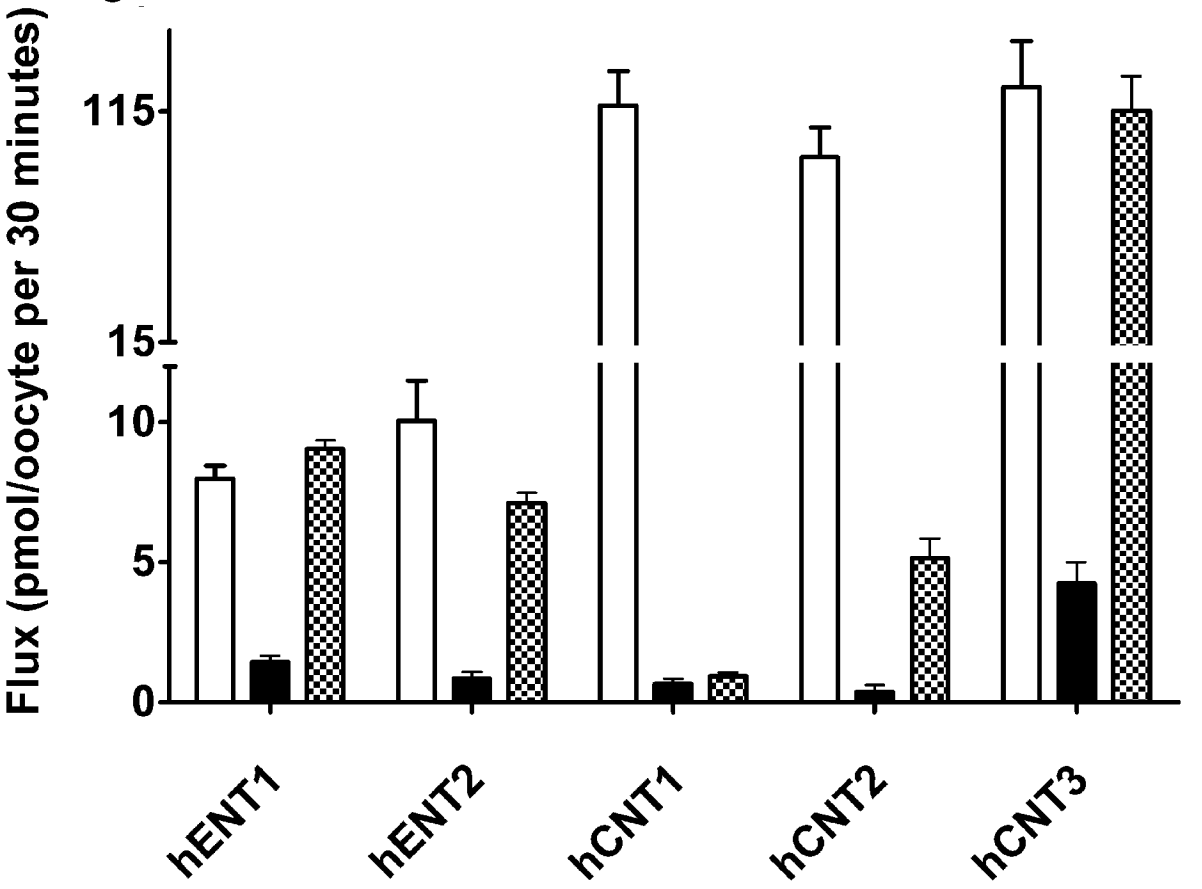
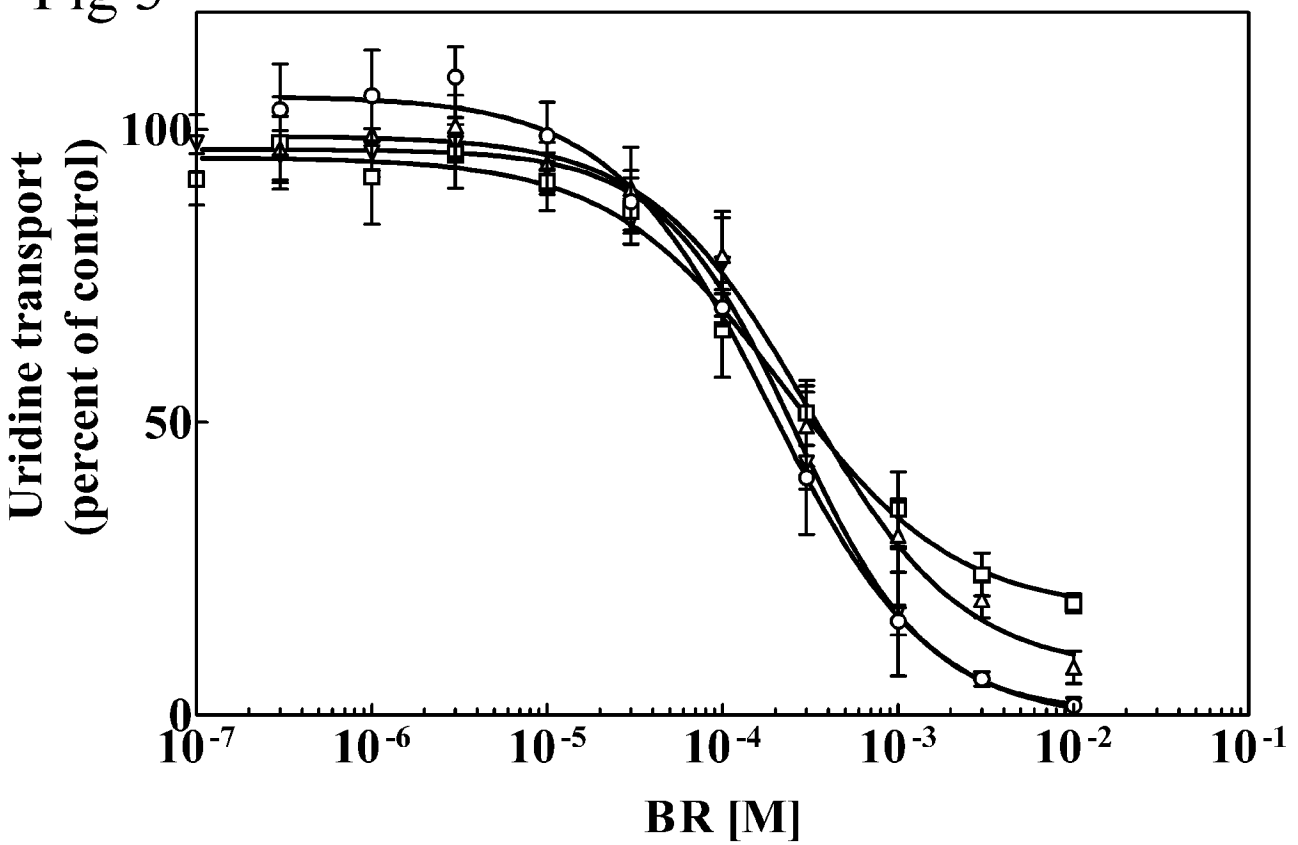
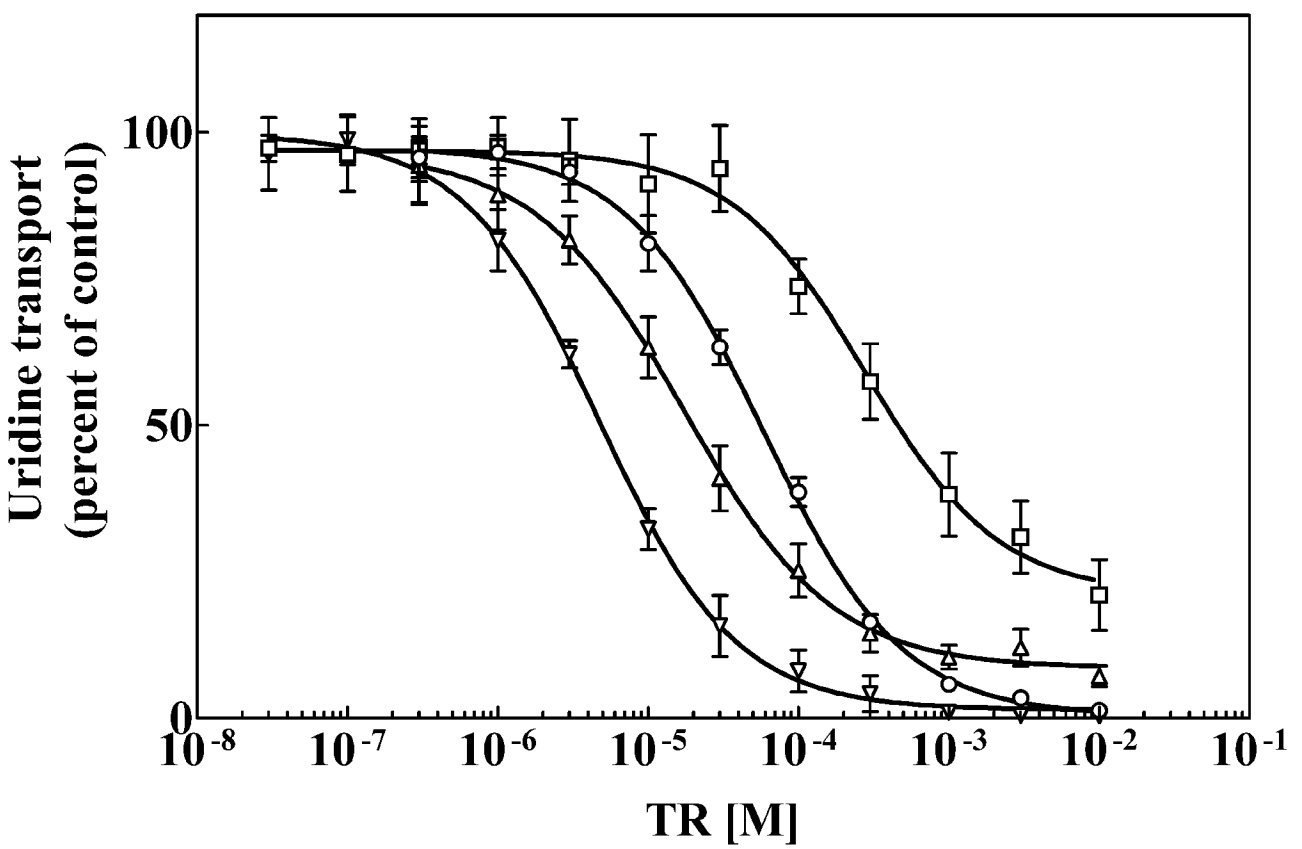

Flux (pmol/oocyte per 30 minutes)
115
15
10
5
0
hENT1
hENT2
hCNT1
hCNT2
hCNT3

Fig 3


Uridine transport
(percent of control)
100
50
0
10-7
10-6
10-5
10-4
10-3
10-2
10-1
BR [M]


Uridine transport
(percent of control)
100
50
0
10-8
10-7
10-6
10-5
10-4
10-3
10-2
10-1
TR [M]