

Role of Human Nucleoside Transporters in the Cellular Uptake of Two Inhibitors of IMP Dehydrogenase, Tiazofurin and Benzamide Riboside

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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 *Xenopus laevis* oocytes and *Saccharomyces cerevisiae* that produced individual recombinant human concentrative nucleoside transporter (CNT) and equilibrative nucleoside transporter (ENT) 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 [³H]uridine transport in *S. cerevisiae* by TR exhibited lower *K_i* values than BR: hCNT3 (5.4 versus 226 μ M),

hENT2 (16 versus 271 μ M), hENT1 (57 versus 168 μ M), and hCNT1 (221 versus 220 μ M). 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 nitrobenzylmercaptapurine ribonucleoside conferred resistance to BR and TR cytotoxicity to hENT1-containing CEM cells, thereby demonstrating the importance of transport capacity for manifestation of cytotoxicity. A breast cancer cell line with mutant p53 exhibited 9-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 whether this finding can be generalized to other cell types.

Inhibitors of IMP 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 ade-

nine dinucleotide and thiazole-4-carboxamide adenine dinucleotide inhibit IMPDH noncompetitively, resulting in cellular depletion of guanylates (GTP and dGTP) and inhibition of the growth of several human tumor cell lines (Jayaram et al., 1982, 1993; Knight et al., 1987). Phase I/II clinical trials with TR in patients with acute myelogenous leukemia showed significant reductions in leukemic cell burden (Tricot et al., 1989; Jayaram et al., 1992; 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 the treatment of chronic myelogenous leukemia in blast crisis (Grifantini, 2000).

In studies with cell lines, TR exhibited 3-fold higher IC₅₀ values than BR (Gharehbaghi et al., 1994b). BR-induced cytotoxicity was attributed to induction of apoptosis and necrosis that was caused by an inhibition of IMPDH and, at higher concentrations, by an induction of DNA double-strand breaks,

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ABBREVIATIONS: IMPDH, IMP dehydrogenase; NBMPR, nitrobenzylmercaptapurine riboside; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; h, human; BR, benzamide riboside; TR, tiazofurin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

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, seems 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). Because 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 μ M and 55 nmol/10⁶ 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 (Griffiths et al., 1997; Crawford et al., 1998; Ritzel et al., 1997, 1998, 2001). The human SLC29 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., 1997, 1998, 2001): 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 *Saccharomyces cerevisiae*) producing individual recombinant human nucleoside transporters were used to examine transportability by measuring inward fluxes of ³H-labeled drugs in the presence or absence of excess nonradioactive 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 ³H-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 down-regulated by p53 tumor suppressor gene (Sherley, 1991; Stadler et al., 1994; Liu et al., 1998).

Materials and Methods

Chemicals. [³H]Benzamide riboside (specific radioactivity, 175 mCi/mmol) was custom-synthesized by American Radiolabeled Chemicals (St. Louis, MO), and [⁵⁻³H]thiazofurin was obtained from

Research Triangle Institute (Research Triangle Park, NC). NBMPR, dilazep, mineral oil, and unlabeled nucleosides were obtained from Sigma Diagnostics Canada (Mississauga, ON, Canada). [5,6-³H]Uridine with a specific activity of 41.2 Ci/mmol was obtained from Moravsek Biochemicals (Brea, CA), and its purity was >97% as confirmed by high-performance liquid chromatography analysis. Tissue culture (96-well) plates, tissue culture medium RPMI 1640 medium, Dulbecco's modified Eagle's medium, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). The CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit was obtained from Promega (Madison, WI). Ecolite was purchased from Valeant Pharmaceuticals (Costa Mesa, CA) and 550 silicone oil from BC Bearings (Edmonton, AB, Canada).

Production of Recombinant Transporters and Measurement of Radioisotope Uptake in Oocytes of *X. laevis*. Production of recombinant human nucleoside transporters in oocytes was performed as described previously (Ritzel et al., 2001). T3 polymerase, T7 polymerase, and SP6 polymerase were used to transcribe linearized plasmids in the presence of the m7GpppG cap, using the MEGAscript transcription system (Ambion, Austin, TX). 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 of RNA transcripts (test samples). Unless otherwise indicated, uptake of [³H]BR and [³H]TR (20 μ M) was measured 3 days after injection. Experiments were performed at room temperature on groups of 10 to 12 oocytes in medium containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM Hepes, pH 7.5. After 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 quantification of oocyte-associated ³H by liquid scintillation counting (LS 6000 IC; Beckman Coulter, Fullerton, CA).

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 performed as described previously (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, 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 used 96-well plates and a semiautomated cell harvester (Micro96 Harvester; Skatron Instruments, Lier, Norway). The relative affinities of the transporters for BR and TR were assessed by measuring their abilities to inhibit the uptake of [³H]uridine. Yeast producing recombinant hENT1, hENT2, hCNT1, or hCNT3 were incubated with graded concentrations of BR and TR in the presence of 1 μ M [³H]uridine for 20 and 10 min, respectively. Each experiment was repeated at least three times. Nonspecifically associated radioactivity was determined in the presence of 10 mM nonradioactive uridine, and these values were subtracted from total uptake values. Data were subjected to nonlinear regression analysis using GraphPad Prism Software version 3.0 (GraphPad Software Inc., San Diego, CA) to obtain IC₅₀ 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 of St. Jude Children's Research Hospital, now at University of Illinois, Chicago, IL) 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 de-

scribed earlier (Lang et al., 2001, 2004). hENT2 stable transfectants were produced by T. Lang (unpublished results) using procedures described previously (Lang et al., 2001, 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 μ M tubercidin, 0.5 μ M cytarabine, and geneticin as described previously (Lang et al., 2001).

Dr. C. Dumontet (Institut National de la Sante et de la Recherche Medicale 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, Rehovot, 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 0.4 mg/ml neomycin. All cultures were kept at 37°C in 5% CO₂/95% air and subcultured every 2 to 3 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). 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/well and exposed to drugs for 4 h. Cells were exposed to graded concentrations of BR (0–50 μ M) or TR (0–500 μ M) and treated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent for assessment of cytotoxicity. IC₅₀ values were calculated from nonlinear 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 Produced in *X. laevis* Oocytes. The transportability of BR and TR (Fig. 1) was examined in the *X. laevis* oocyte expression system by comparing the uptake of 20 μ M [³H]BR and [³H]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.

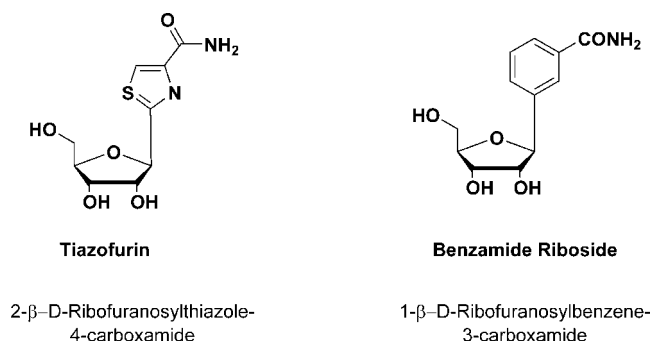


Fig. 1. Structures of TR and BR.

[³H]Uridine was included as a control permeant. The mediated fluxes (picomole per oocytes per 30 min) of TR were generally higher than those of BR: hENT1 (9 versus 1.5), hENT2 (7 versus 0.85), hCNT1 (0.9 versus 0.7), hCNT2 (5 versus 0.4), and hCNT3 (115 versus 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 greater than 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 [³H]TR and [³H]BR by hENTs and hCNTs Produced in *S. cerevisiae*. Time courses for influx of [³H]uridine, [³H]TR, and [³H]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 μ M [³H]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 the addition of 10 mM unlabeled uridine to assay mixtures. Transportability of 1 μ M [³H]TR and [³H]BR was assessed in the presence or absence of excess unlabeled 10 mM uridine. Mediated transport of 1 μ M TR by hENT1 and hENT2 was higher than that of BR. Uptake of [³H]uridine, [³H]BR, and [³H]TR by hENT1 was blocked by the addition of 10 mM nonradioactive uridine (data not shown). TR exhibited higher initial rates of transport (measured in picomoles per milli-

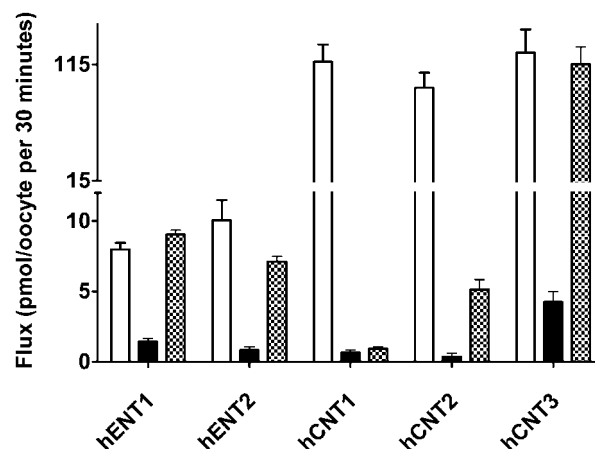


Fig. 2. Transport of BR, TR, and uridine by recombinant human equilibrative (ENT) and concentrative (CNT) nucleoside transporters produced in *X. laevis* oocytes. Oocytes were injected with either 20 nl of water alone or that containing 20 ng of RNA transcripts. Uptake of 20 μ M [³H]uridine (\square), [³H]BR (\blacksquare), and [³H]TR (\hatched) during 30-min exposures at 20°C was determined after 3 days in NaCl-containing transport medium. Each value represents the mean of 10 to 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 picomoles per oocyte per 30 min. The fluxes (mean \pm S.E.M.) for TR and BR are in the following order: hCNT3 (115 \pm 15 versus 4.3 \pm 0.7), hENT1 (9 \pm 0.3 versus 1.5 \pm 0.2), hENT2 (7 \pm 0.4 versus 0.85 \pm 0.25), hCNT2 (5.2 \pm 0.7 versus 0.38 \pm 0.24), and hCNT1 (0.93 \pm 0.13 versus 0.66 \pm 0.18), respectively.

grams of protein per minute) than BR in yeast-producing hCNT3 (89 versus 0.8), hENT1 (20 versus 3), and hENT2 (7.7 versus 0.5), but not hCNT1 (0.06 versus 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 [³H]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 μ M [³H]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 IC₅₀ 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 whether the presence of nucleoside transport activity was important for the 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, 2004). The IC₅₀ values generated by exposing cells to graded concentrations of either BR (0–50 μ M) or TR (0–500 μ M) 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 μ M NBMPR for either 4 or 48 h. Cells were first incubated for 1 h with NBMPR to ensure complete blockade of the hENT1-mediated process before the addition of graded concentrations of drugs. BR was more cytotoxic than TR during both exposure conditions. The pro-

TECTIVE effects of NBMPR were approximately 4-fold for BR but were much smaller for TR-mediated cytotoxicity (Table 3). These results demonstrated that NBMPR protected cells against both BR- and TR-mediated cytotoxicity during drug

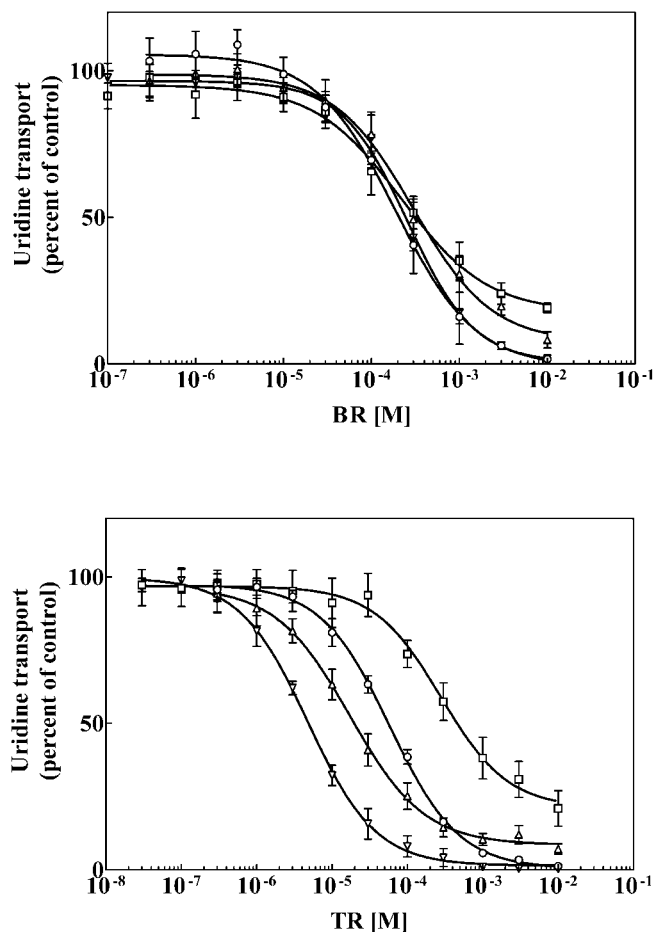


Fig. 3. Inhibition of recombinant hENT1-, hENT2-, hCNT1-, and hCNT3-mediated uptake of uridine by BR and TR. Yeast cells expressing pY-PhENT1 (\circ), pYPhENT2 (Δ), pYPhCNT1 (\square), or pYPhCNT3 (∇) were incubated for 20 min (10 min for hCNT3) in the presence of 1 μ M [³H]uridine with graded concentrations of BR (A) or TR (B). Uridine transport rates (mean \pm S.E.M., $n = 4$) in the presence of inhibitor are represented as percentages of the rates observed in the absence of inhibitor (control), and S.E.M. values are not presented where the size of the point is larger than the S.E.M. Three separate experiments gave similar results. IC₅₀ 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 μ M [³H]-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 under *Materials and Methods*. Initial rates (in picomoles per milligram of protein per minute) \pm S.E. of uptake were calculated from the slopes of the linear regression lines using GraphPad Prism software version 3.02; rates in the presence of 10 mM nonradioactive uridine were subtracted from rates in its absence to give rates of mediated uptake (i.e., transport).

Recombinant Transporter	Uridine	BR	TR
		<i>pmol / mg protein / min</i>	
hENT1	21.5 \pm 1.1	3 \pm 0.3	20 \pm 0.7
hENT2	16.6 \pm 1.02	0.5 \pm 0.3	7.7 \pm 0.2
hCNT1	7.9 \pm 0.2	0.2 \pm 0.05	0.06 \pm 0.03
hCNT3	374 \pm 19	0.8 \pm 0.02	89 \pm 4

TABLE 2

IC₅₀ and K_i values for BR and TR inhibition of [³H]uridine transport in yeast producing human nucleoside transporters

Rates of transport of 1 μ M [³H]uridine into yeast expressing pYPhENT1, pY-PhENT2, pYPhCNT1, or pYPhCNT3 were measured over 20 or 10 min in the presence of graded concentrations of BR and TR as described in Fig. 3. Mean IC₅₀ \pm S.E. values were determined using GraphPad Prism software version 3.0 and then converted to K_i values by the Cheng and Prusoff (1973) equation using K_m values for uridine (Visser et al., 2002, 2004; Zhang et al., 2003).

Recombinant Transporter	BR		TR	
	IC ₅₀	K_i	IC ₅₀	K_i
	μ M			
hENT1	172 \pm 21	168 \pm 21	58 \pm 5	57 \pm 5
hENT2	272 \pm 42	271 \pm 42	16 \pm 3	16 \pm 3
hCNT1	224 \pm 41	220 \pm 40	225 \pm 45	221 \pm 44
hCNT3	252 \pm 46	226 \pm 41	6 \pm 0.7	5.4 \pm 0.6

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 ($>500 \mu M$) both in the presence and absence of NBMPR. In addition, at higher concentrations of BR and TR, diffusion processes may overcome the mediated processes. Cytotoxicity experiments with cells possessing hCNT3 were not undertaken because of 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. Because p53 mutation is the most commonly observed step in tumorigenesis (Soussi et al., 2000), inhibitors of IMPDH, such as BR and TR, may be useful as anti-tumor agents. To test the effects of BR and TR on cytotoxicity toward 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 4 h, and cytotoxicity was assessed with the MTS reagent. Exposure to TR had no effect on either cell line at concentrations as high as $500 \mu M$, whereas BR was approximately 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 (Jayaram et al., 1982; Gharehbaghi et al., 1994a), an enzyme that is required for de novo synthesis of guanine nucleotides. Both drugs are active in various preclinical model systems (Tricot et al., 1989; Jayaram et al., 1992; Wright et al., 1996; Rauko et al., 2001), 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 (Jayaram et al., 1999; Jager et al., 2002), relatively little is known about their routes of mem-

brane 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 (Damaraju et al., 2003; Baldwin et al., 2004). 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. Because 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, because the distribution of nucleoside transporter types differs among normal and neoplastic tissues in humans (Ritzel et al., 1997, 1998, 2001). We 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 *X. laevis* oocytes, and the transportability of [3H]BR and [3H]TR was studied. Although rates of transport of [3H]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 and hENT1 and, to a much lesser extent, hENT2 and hCNT1.

The initial rates of uptake of [3H]TR and [3H]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 because it was inhibited by excess nonradioactive uridine. The relative binding affinities of recombinant hENT1, hENT2, hCNT1, and hCNT3 for TR and BR were determined by examining their ability to inhibit [3H]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 (Table 3), although at the highest concentrations, BR and TR were cytotoxic to all cell lines, probably

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 under *Materials and Methods*. In protection experiments, CEM cells were treated with $1 \mu M$ NBMPR 1 h before the addition of BR or TR in the presence of $1 \mu M$ 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 \pm S.E. values were determined using GraphPad Prism software version 3.0.

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

^a CEM has endogenous hENT1 activity.

^b CEM-ARAC 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 4 h to graded concentrations of BR (0–50 μM) and TR (0–500 μM) and assessed by the MTS assay as described under *Materials and Methods*. IC_{50} values obtained are means of three experiments, each conducted with six replicates. Mean \pm S.E. values were determined using GraphPad Prism software version 3.0.

Drug	MN-1 IC_{50}	MDD-2 IC_{50}
	μM	
BR	208 \pm 23	23 \pm 4.7
TR	>500	>500

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 4-fold protection against cytotoxicity with BR during both short and long exposures (Table 3).

It has been shown that IMPDH is downstream of p53 (Sherley, 1991; Sherley et al., 1995; Liu et al., 1998). 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 9-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 μ M 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 whether the activity of IMPDH inhibitors in p53 mutant cells is a generalizable finding. Such studies may lead to the use of IMPDH inhibitors in the treatment of tumors with mutated p53 that exhibit resistance to conventional anticancer nucleoside drugs such as gemcitabine, cladribine, and fludarabine (Pettitt et al., 1999; Galmarini et al., 2002, 2003).

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 with 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 whether p53 mutant cells are a potential pharmacological target of inhibitors of IMPDH.

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