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Maintenance of Differential Methotrexate Toxicity between Cells Expressing Drug-Resistant and Wild-Type Dihydrofolate Reductase Activities in the Presence of Nucleosides through Nucleoside Transport Inhibition

*Christopher A. Warlick, Colin L. Sweeney and R. Scott McIvor**

Gene Therapy Program, Institute of Human Genetics, and Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455, U.S.A.

ABSTRACT. Methotrexate (MTX), a potent inhibitor of dihydrofolate reductase (DHFR), has been used widely as a chemotherapeutic agent and as a selective agent for cells expressing drug-resistant DHFR activity. MTX deprives rapidly dividing cells of reduced folates that are necessary for thymidylate synthesis and *de novo* purine nucleotide synthesis. However, MTX toxicity can be circumvented by salvaging thymidine (TdR) and purine nucleosides. Here we have investigated conditions under which nucleoside transport inhibition can be used to maintain differential MTX toxicity between unmodified cells and cells expressing drug-resistant DHFR activity in the presence of exogenous nucleosides. PA317 cells (a 3T3 derivative cell line) were rescued from the toxicity of 0.1 μM MTX by 1.0 μM TdR in the presence of 100 μM inosine. The nucleoside transport inhibitor dipyridamole (DP) resensitized these cells to MTX, even in the presence of exogenous nucleosides. Furthermore, PA317 cells transduced with any of three retroviruses encoding drug-resistant DHFRs remained resistant to MTX over all concentrations tested (up to 10.0 μM) in the presence of DP. Similar results were obtained in transduced HuH7 and K562 cell lines, a human hepatoma and a human leukemia cell line, respectively. We conclude that nucleoside transport inhibition increases the toxicity and selectivity of MTX in cultured cells, and therefore is an effective way to maintain differential MTX toxicity between unmodified and DHFR-modified cells. Our results support the use of nucleoside transport inhibition in *in vivo* selection protocols involving the liver and hematopoietic systems. BIOCHEM PHARMACOL **59**;2:141–151, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. dihydrofolate reductase; methotrexate; drug resistance; nucleoside transport inhibitors; dipyridamole; chemotherapy

DHFR† in mammals catalyzes the NADPH-dependent reduction of folate to dihydrofolate, and dihydrofolate to tetrahydrofolate. Rapidly dividing cells are reliant upon reduced folates as co-factors for enzymes involved in thymidylate synthesis, *de novo* purine biosynthesis, and glycine synthesis. MTX, a potent competitive inhibitor of DHFR, is toxic for rapidly dividing cells, making it a commonly used, clinically effective chemotherapeutic agent.

DHFRs bearing amino acid substitutions rendering them resistant to antifolates have been used extensively *in vitro* for dominant selection in mammalian cells [1–4] and also have been used *in vivo* for protection of experimental animals from the toxicity of antifolates [5–10]. MTX selection requires toxicity of the drug for normal cells not expressing drug-resistant DHFR activity. However, cells are able to circumvent the toxicity of MTX and other anti-metabolites by salvaging exogenous purines and TdR [11–13]. The resulting insensitivity to MTX mitigates the selective advantage afforded by drug-resistant DHFR expression in the presence of MTX. Blocking the uptake and incorporation of nucleic acid precursors into nucleotide pools prevents the ability of these agents to rescue cells from MTX toxicity. For this purpose, nucleoside transport inhibitors have been tested as adjuncts to anti-metabolites such as MTX for increasing their toxicity and creating a more potent chemotherapeutic and selective effect both *in vitro* [12–17] and *in vivo* [12, 13, 17, 18].

In this study, we set out to define conditions whereby differential MTX toxicity between unmodified cells and cells expressing drug-resistant DHFRs can be maintained in the presence of nucleosides through the inhibition of nucleoside transport. We found that the rescue of unmodified cells from antifolate toxicity was dependent upon

* Corresponding author: Dr. R. Scott McIvor, Institute of Human Genetics, University of Minnesota, Box 206 FUMC, 420 Delaware St. SE, Minneapolis, MN 55455. Tel. (612) 626-1497; FAX (612) 626-7031; E-mail: mcivo001@maroon.tc.umn.edu

† *Abbreviations*: DHFR, dihydrofolate reductase; DMEM, Dulbecco's modified Eagle medium; DP, dipyridamole; LTR, long terminal repeat; MTX, methotrexate; NBMPR-P, nitrobenzylmercaptopurine ribose-5′-monophosphate; TdR, thymidine; tk, thymidine kinase; and WT, wild-type.

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exogenous nucleoside concentration, and that this rescue was abrogated by transport inhibition using DP. These results define conditions in which selection of DHFR-modified cells is impaired by salvageable nucleosides and support the use of nucleoside transport inhibition as a means of restoring effective conditions for selection. Thus, under these conditions, nucleoside transport inhibition can be applied for improved selectivity of DHFR-expressing cells in both *in vitro* and *in vivo* settings.

MATERIALS AND METHODS

Mammalian Cell Culture

The murine fibroblast cell line NIH 3T3 tk^- [19], the ecotropic retrovirus packaging cell line GP+E86 [20], the amphotropic retrovirus packaging cell line PA317 [21], and the human hepatoma cell line HuH7 [22] were maintained in DMEM (Gibco BRL) supplemented with either 10% newborn calf serum (3T3, GP+E86, and PA317 cells) or 10% fetal bovine serum (HuH7 cells) (Summit Biotechnology). The human chronic myelogenous leukemia cell line K562 [23, 24] was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. All media were supplemented with 2 mM glutamine (Sigma), 50 U/mL of penicillin, 50 μg/mL of streptomycin, and 0.125 μg/mL of Fungizone (Gibco BRL), and all cells were maintained in a humidified atmosphere at 37° and 5% CO_2. MTX toxicity experiments in HuH7 and K562 cells were conducted using medium containing 10% newborn calf serum instead of fetal bovine serum.

Retrovirus Producer Cell Lines

Plasmids encoding retroviral vectors expressing variant DHFRs were generated using standard techniques [25] and then packaged by transfection into GP+E86 cells using the DNA calcium phosphate co-precipitation technique [26]. Transient virus-containing supernatant was then shuttle packaged into PA317 cells, subsequently selecting clones in 0.25 μM MTX and screening for those producing the highest titer of DHFR transducing virus. Virus titer was determined by infecting 3T3 tk^- cells overnight in the presence of 8 μg/mL of polybrene and then subculturing the cells into medium containing 0.25 μM MTX the next day. The cells were maintained in selective medium for approximately 2 weeks, at which time the number of MTX-resistant colonies was determined.

Transductions of HuH7 and K562 Cells

HuH7 and K562 cells were transduced with amphotropic retrovirus encoding either the Arg22, Tyr22, or Ser31 variant DHFRs at a multiplicity of infection between 0.1 and 40 in the presence of 8 μg/mL of polybrene. Cells were exposed to virus overnight and subcultured 1:10 the next day into selective media containing 0.3 μM MTX and 5 μM DP. DP was included to reduce the background growth observed in control, uninfected cultures when MTX selections were carried out in the presence of fetal bovine serum. Cells were maintained in selective media for approximately 2 weeks. Individual plates containing multiple drug-resistant clones of HuH7 cells were trypsinized and replated, establishing polyclonal populations of transduced cells.

Cell Viability Assay

Cell viability assays were conducted using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega) as per the manufacturer's instructions. Briefly, 1500 cells/well were inoculated into flat-bottom 96-well plates (Corning). Combinations of MTX, nucleosides, and nucleoside transport inhibitors were added to a final volume of 100 μL with a multi-pipettor (Lab Systems) immediately after cell plating. Cells were then maintained for 4 days at 37° in a humidified atmosphere with 5% CO_2. On day 4, 20 μL of a tetrazolium indicator solution consisting of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) was added to each well and incubated with the cells for 2 hr. Absorbance at 492 nm was then determined using a Bio-Rad 2550 enzyme immunoassay plate reader. Unless indicated otherwise, cell viability is expressed as a percentage of that observed for a set of unsupplemented control cells cultured in parallel. Results are reported as the means of three determinations. With rare exceptions, the SD was less than 20%.

TdR Uptake Assay

Cells were plated in 6-well tissue culture dishes in either DMEM supplemented with 10% newborn calf serum (PA317 and HuH7) or RPMI 1640 supplemented with 10% newborn calf serum (K562 cells). The next day, 5 μCi of [*methyl*-^{3}H]TdR (Amersham Lifescience) alone or [*methyl*-^{3}H]TdR plus DP was added to each well in a final volume of 1.0 mL (final TdR concentration of 1 μM, final DP concentration of 5 μM). The cells were then incubated at 37°, and at various time points thereafter were washed three times with room temperature PBS, and lysed in 1 mL of digestion buffer (100 mM NaCl, 10 mM Tris, 25 nM EDTA, 0.5% SDS, and 0.1 mg/mL of proteinase K) for 15 min at 37°. At this point, the radioactivity was determined using a Beckman LS 1801 scintillation counter (Beckman) with Ecolume scintillant (ICN). Results are reported as the means of at least three samples.

Southern Hybridization Analysis

DNA was extracted from transduced PA317, HuH7, and K562 cells for molecular analysis by lysing the cells in digestion buffer at 37° overnight. Lysates were extracted with a 1:1 mixture of phenol and chloroform, and then total nucleic acids were precipitated in 2 vol. of ethanol. Ten micrograms of DNA from each cell population was

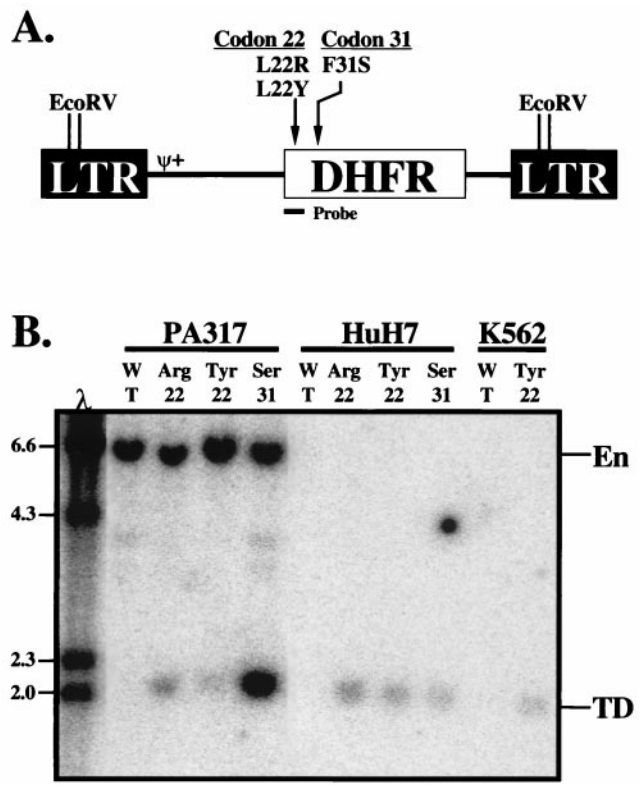

FIG. 1. DHFR retroviral constructs and packaging. (A) DHFR retrovirus constructs. Substitutions in the *DHFR* gene that were used in this study are indicated above the arrows. The 5′ LTR and ψ + sequences (up to the *Bam*HI cloning site) were derived from pLXSN [27], and the 3′ LTR sequences downstream of DHFR (starting with *Cla*I) were from pLNCX [27]. The Arg22 DHFR coding region (from *Bam*HI to *Cla*I) was obtained from pMoDMo [28], except that a sequence between *Sal*I and *Sph*I derived from the pUC19 polylinker was deleted to eliminate an upstream ATG [29]. The Tyr22 and Ser31 DHFR coding sequences (*Hin*dIII to *Nco*I) were obtained from pSV-DHFR expression plasmids [30]. The variant DHFR-encoding retroviral constructs were shuttle packaged into PA317 cells for concentration–response studies (see text) and as a source of amphotropic retrovirus to transduce HuH7 and K562 cells. The locations of the *Eco*RV sites as well as the probe used for Southern analysis (Fig. 1B) are indicated. (B) Southern blot of DNA extracted from untransduced (WT) and transduced cell lines. The indicated samples were digested with *Eco*RV and probed with a fragment from the murine *DHFR* gene indicated in Fig. 1A (see also Materials and Methods). The two internal *Eco*RV sites of the transduced retroviral integrant generate a 2.1-kb hybridizing fragment indicated by "TD." A 6.5-kb hybridizing fragment from the endogenous murine *DHFR* gene is indicated by "En." The murine *DHFR* probe used does not detect human endogenous DHFR sequences. "λ," λ *Hin*dIII DNA with the size of each band (in kb) indicated on the left.

digested overnight with an excess of *Eco*RV (Boehringer Mannheim), resolved by electrophoresis on 0.8% agarose, transferred onto a nylon membrane (Nytran Plus, Schleicher & Schuell), and then probed with a radiolabeled (Oligolabeling Kit, Pharmacia Biotech) 0.4-kb polymerase chain reaction (PCR) product from the murine *DHFR* gene (see Fig. 1 for location of probe) as previously described [9].

Chemicals

MTX was obtained as the sodium salt solution (Lederle Parenterals) and diluted in culture medium. TdR, inosine, DP, and polybrene were obtained from the Sigma Chemical Co., solubilized in water or acidified PBS (DP), and diluted in culture medium.

RESULTS

Protection of PA317 Cells from MTX Toxicity by Expression of Drug-Resistant DHFR Activity

The purpose of this study was to characterize *in vitro* the conditions under which nucleoside transport inhibition could be used to maintain differential MTX toxicity between unmodified cells and cells expressing MTX-resistant DHFR activity in the presence of nucleosides. To introduce and express drug-resistant DHFR activity in different cell types, retroviral vectors were constructed in which the Arg22, Tyr22, and Ser31 mutant DHFRs were regulated transcriptionally by the Moloney murine leukemia virus LTR (Fig. 1A). These retroviral constructs were shuttle packaged into the amphotropic retroviral producer cell line PA317 as described in Materials and Methods, selecting stable integrants in 0.25 μM MTX. To verify the presence of the retroviral integrant, DNA was extracted from these cells for Southern hybridization analysis as described in Materials and Methods, digesting with *Eco*RV and probing with a 0.4-kb PCR fragment from the murine *DHFR* gene [9] (Fig. 1B). All three DHFR virus producer cell lines contained a 2.1-kb DHFR-hybridizing fragment diagnostic for the retroviral integrant. These cell lines were used subsequently in the concentration–response experiments described below, and also provided a source of amphotropic retrovirus to transduce mutant DHFRs into K562 and HuH7 cells for concentration–response studies.

To assess resistance to MTX toxicity conferred by drug-resistant DHFR expression in PA317 cells, viability studies were conducted over a large range of MTX concentrations. Untransduced and transduced PA317 cells were incubated for 4 days with MTX at concentrations ranging from 0.001 to 10.0 μM. Cell viability was assessed by using an MTS colorimetric assay at 492 nm. We found that untransduced PA317 cell viability was reduced to less than 10% of controls at 0.3 μM MTX (Fig. 2A). In contrast, PA317 cells transduced with Tyr22, Arg22, or Ser31 DHFRs maintained greater than 90% viability throughout the entire range of MTX concentrations tested. These results indicate a differential sensitivity of at least 1000-fold between untransduced PA317 cells and the tested DHFR-virus transduced cells.

Rescue of PA317 Cells from MTX Toxicity by Exogenous Nucleosides

The MTX toxicity observed in untransduced PA317 cells in the presence of MTX presumably is due to inhibition of

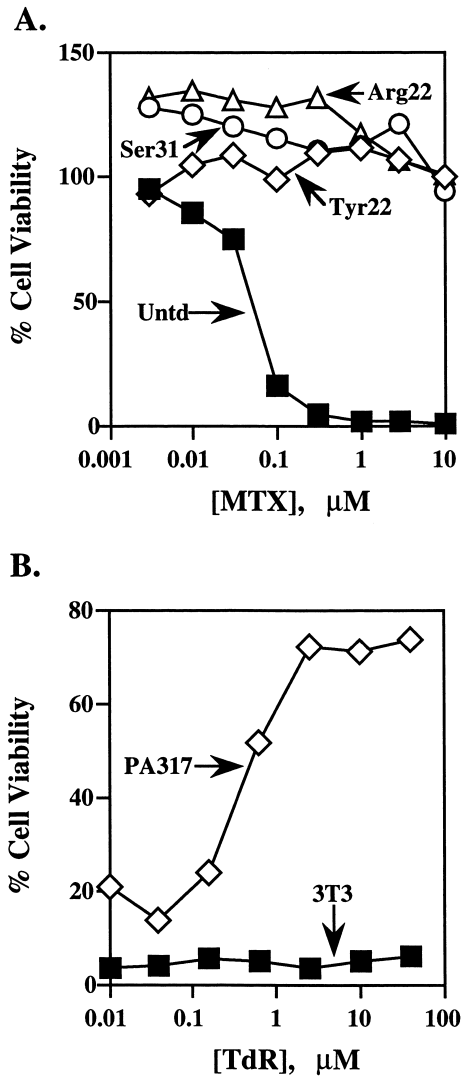

FIG. 2. MTX toxicity and nucleoside rescue in PA317 cells. (A) MTX concentration–response relationship in PA317 cells. The indicated cell populations were tested in a 4-day growth assay at various concentrations of MTX. Experiments were performed in triplicate as described in Materials and Methods. Growth is expressed as a percentage of that observed for an unsupplemented culture of the same cells. Untd, untransduced cells; Arg22, Tyr22, and Ser31 indicate cells transduced by retroviruses encoding variant DHFRs. (B) TdR concentration–response relationship in PA317 and 3T3 tk$^-$ cells. Cells were cultured in medium containing 0.1 μM MTX, 100 μM inosine, and various amounts of TdR in a 4-day growth assay. Experiments were performed in triplicate as described in Materials and Methods.

DHFR, depleting cells of tetrahydrofolate derivatives. However, salvage of exogenous nucleosides can circumvent this MTX toxicity [11, 14]. To characterize the conditions in our system under which untransduced PA317 cells could be rescued by nucleosides, cells were plated in the presence of 0.1 μM MTX, 100 μM inosine as a source of purines (a presumed saturating concentration), and various amounts of TdR. TdR levels $\geq$ 1 μM rescued the PA317 cells from the toxic effects of MTX (Fig. 2B). The presumed mechanism of this rescue is the uptake of exogenous nucleosides provided from the medium, thus circumventing MTX inhibition of DHFR and providing an alternate source of nucleotide precursors.

For cells to utilize imported TdR, it must be phosphorylated by thymidine kinase to TdR monophosphate. PA317 cells were originally derived from thymidine kinase-deficient NIH 3T3 cells by transfection with a plasmid designed for expression of the herpes simplex virus thymidine kinase [21], thus providing these cells with the capability to metabolize imported TdR. In contrast, we found that 3T3 cells deficient in thymidine kinase activity were not rescued from MTX toxicity by TdR at any concentration (Fig. 2B). Thus, import and subsequent phosphorylation of TdR were necessary for the rescue of untransduced PA317 cells, implying that if either one of these steps is prevented, then MTX sensitivity can be restored despite the presence of exogenous nucleosides.

Restoration by DP of Differential MTX Toxicity between PA317 and Variant DHFR-Expressing PA317 Cells in the Presence of Exogenous Nucleosides

We investigated the use of nucleoside transport inhibition as a means of resensitizing cells to MTX in the presence of exogenous nucleosides. To ensure that DP blocked nucleoside uptake in our system, PA317 cells were cultured in the presence of 5 μCi of [^{3}H]TdR with or without 5 μM DP for 1–4 hr at 37°, and then [^{3}H]TdR uptake was determined. [^{3}H]TdR uptake in PA317 cells was inhibited substantially by DP (Fig. 3A). Next, untransduced and transduced PA317 cells were incubated with 0.3 μM MTX, 100 μM inosine, 1.0 μM TdR, and various amounts of DP (ranging from 0.01 to 100 μM). Indeed, untransduced PA317 cells were resensitized to MTX in the presence of exogenous nucleoside levels previously shown to rescue cells from MTX toxicity (Fig. 4A). Conversely, cells expressing mutant DHFRs remained largely resistant to MTX and DP. The largest differential between untransduced PA317 cells and cells transduced with any of the three DHFRs tested was observed consistently at 5 μM DP, a level that was not toxic for cells in the absence of MTX (data not shown). Cells expressing the Arg22 variant DHFR were more sensitive to DP in the presence of MTX than cells expressing either Ser31 or Tyr22 variant DHFRs.

PA317 cells exposed to various concentrations of MTX in the presence of 100 μM inosine, 1.0 μM TdR, and 5 μM DP exhibited a concentration–response relationship similar to that observed in the absence of exogenous nucleosides (Fig. 4B). In contrast, the Ser31 and Tyr22 DHFR-transduced cells remained resistant to MTX over all concentrations tested. Arg22-expressing cells were more sensitive to MTX in the presence of 5 μM DP, perhaps due to the extremely low level of catalytic activity associated with the Arg22 mutation [30, 31]. Inhibition of nucleoside uptake by incubation with 5 μM DP thus restored differential MTX toxicity between untransduced and DHFR virus-transduced PA317 cells in the presence of nucleosides that otherwise rescue cells from MTX.

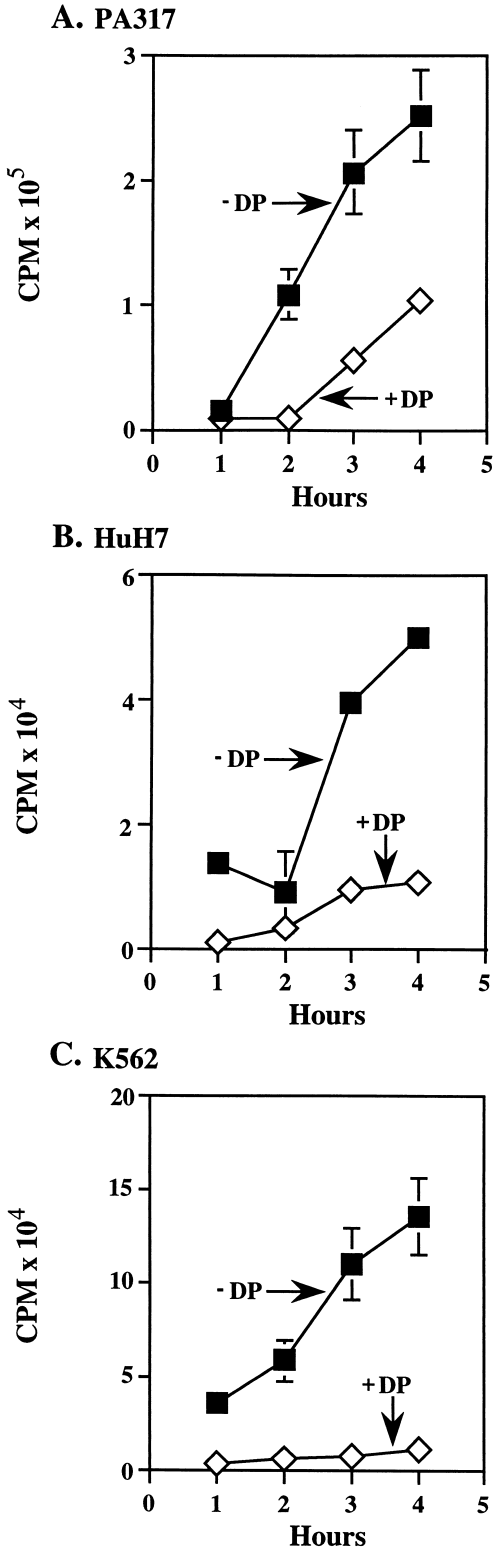

FIG. 3. Effect of DP on [^{3}H]TdR uptake. Cells were incubated in medium containing 5 μCi of [^{3}H]TdR with or without 5 μM DP for 1–4 hr, washed and lysed. Radioactivity (cpm) was determined by liquid scintillation counting. Each point represents the mean ± SD of three trials. (A) PA317 (2 × 10^5 cells/well). (B) HuH7 (1.5 × 10^5 cells/well). (C) K562 (2 × 10^5 cells/well).

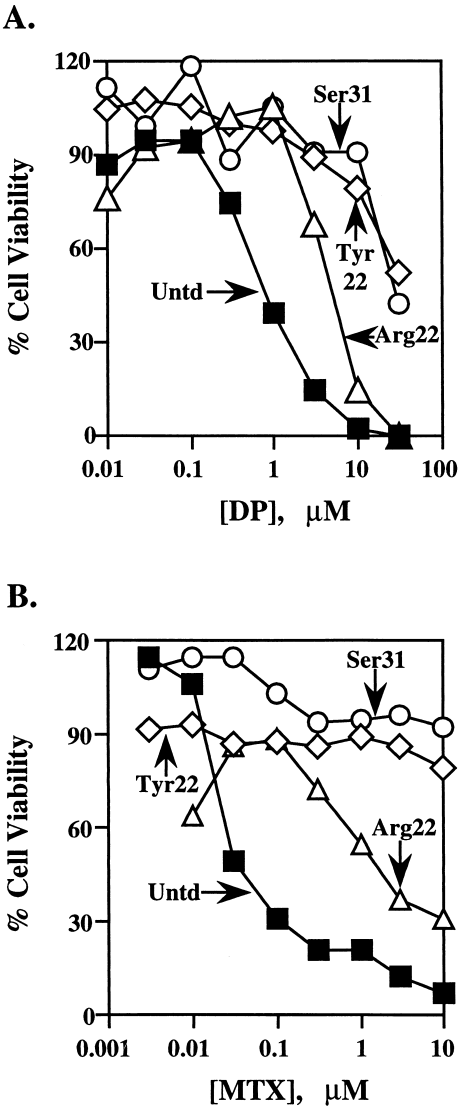

FIG. 4. Restoration of differential toxicity between untransduced and DHFR virus-transduced PA317 cells by DP. (A) DP concentration–response relationship. Cells were cultured in medium containing 0.3 μM MTX, 100 μM inosine, 1.0 μM TdR, and various amounts of DP. Viability is expressed as a percentage of the viability of cells cultured in 0.3 μM MTX, 100 μM inosine, and 1.0 μM TdR in the absence of DP. Experiments were performed in triplicate as described in Materials and Methods. (B) MTX concentration–response relationship in the presence of nucleosides and DP. Cells were cultured in medium containing 100 μM inosine, 1.0 μM TdR, 5 μM DP, and various amounts of MTX. Untd, untransduced cells; Arg22, Tyr22, and Ser31 indicate cells transduced by retroviruses encoding variant DHFRs. Experiments were performed in triplicate as described in Materials and Methods.

Protection of HuH7 and K562 Cells from MTX Toxicity by Expression of Drug-Resistant DHFR Activity

To extend our studies of differential MTX toxicity to other relevant cell types, we used DHFR virus generated from the PA317 producer cell lines described above to transduce HuH7 cells and K562 cells as representative liver and hematopoietic cell lines, respectively (described in Materi-

als and Methods). Total nucleic acid was harvested from the transduced HuH7 and K562 cell populations and subjected to Southern hybridization analysis. The presence of the 2.1-kb DHFR-hybridizing fragment verified the presence of retroviral integrants in the selected cell populations (Fig. 1B).

MTX concentration–response studies were conducted on HuH7 cells transduced with Arg22, Tyr22, or Ser31 variant DHFRs, and on K562 cells transduced with the Tyr22 variant DHFR. We found that HuH7 cells expressing any of the three drug-resistant DHFRs tested were largely resistant to MTX over all concentrations tested, while untransduced HuH7 cells were reduced to approximately 20% of control values at MTX concentrations above 0.1 μM (Fig. 5A). K562 cell viability was reduced to less than 10% at MTX concentrations above 0.03 μM, whereas Tyr22 DHFR-transduced K562 cells maintained 90% viability in 0.1 μM MTX (Fig. 5B). At higher MTX concentrations, the transduced K562 cells exhibited reduced viability.

Maintenance of Differential MTX Toxicity between Normal and Variant DHFR-Expressing HuH7 and K562 Cells in the Presence of Nucleosides

To characterize the conditions under which exogenous nucleosides rescued HuH7 and K562 cells from MTX toxicity, untransduced HuH7 and K562 cells were exposed to 0.3 μM MTX, 100 μM inosine, and various amounts of TdR. As observed for PA317 cells, both HuH7 and K562 cells were rescued from MTX toxicity at levels of TdR greater than 1.0 μM (Fig. 6A). Interestingly, HuH7 cells were mostly protected from MTX toxicity when incubated with 0.3 μM MTX and 10 μM TdR in the absence of exogenous inosine (data not shown), implying some difference in the folate requirement for purine biosynthesis in this cell line. These conditions provided little protection for K562 cells, which required inosine for rescue. One hundred micromolar inosine alone provided no rescue for either cell line (data not shown).

We investigated whether nucleoside transport inhibition could be used to resensitize HuH7 and K562 cells to MTX in the presence of exogenous nucleosides, as demonstrated above for PA317 cells. To confirm that DP inhibited nucleoside uptake in these cells, they were incubated with [^{3}H]TdR in the presence or absence of 5 μM DP for up to 4 hr. Similar to our findings with PA317 cells, uptake of [^{3}H]TdR into HuH7 and K562 cells was inhibited substantially by 5 μM DP (Fig. 3B and C). Untransduced HuH7 and K562 cells were then cultured in the presence of 0.3 μM MTX, 100 μM inosine, 1.0 μM TdR, and DP at concentrations varying between 0.01 and 30.0 μM. Maximum resensitization to MTX was observed at concentrations of DP between 5 and 10 μM, reducing cell viability to less than 40% of controls for HuH7 cells and to less than 10% for K562 cells (Fig. 6B).

Finally, MTX concentration–response studies were conducted using DHFR virus-transduced and untransduced

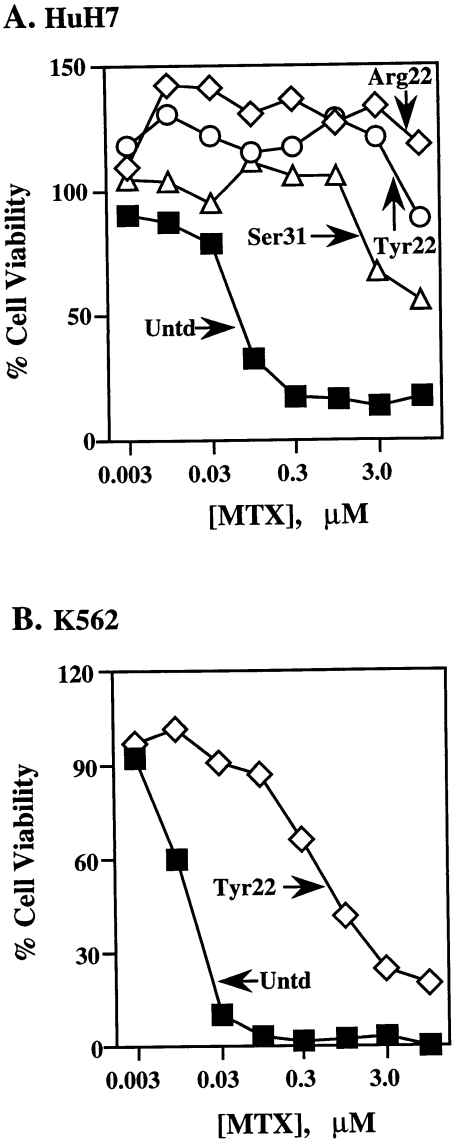

FIG. 5. MTX concentration–response relationship in HuH7 and K562 cells. Untransduced cells or cells transduced with DHFR virus were tested in a 4-day growth assay at various concentrations of MTX. Viability is expressed as a percentage of that observed in an unsupplemented culture of the same cell population. Untd, untransduced cells; Arg22, Tyr22, and Ser31 indicate cells transduced with various variant DHFRs. Experiments were performed in triplicate as described in Materials and Methods.

HuH7 and K562 cells in the presence of 100 μM inosine, 1.0 μM TdR, and 5.0 μM DP (Fig. 7). We found that DP restored the MTX concentration–response character observed for untransduced HuH7 cells in the absence of nucleosides, whereas the transduced cells remained resistant to MTX up to 10.0 μM, the highest concentration tested (Fig. 7A). DP also resensitized untransduced K562 cells to MTX in the presence of nucleosides (Fig. 7B), although the Tyr22 DHFR transduced cells exhibited sensitivity to MTX at concentrations above 1.0 μM, as when incubated in the absence of DP and nucleosides (Fig. 5B). DP thus restored the differential MTX toxicity be-

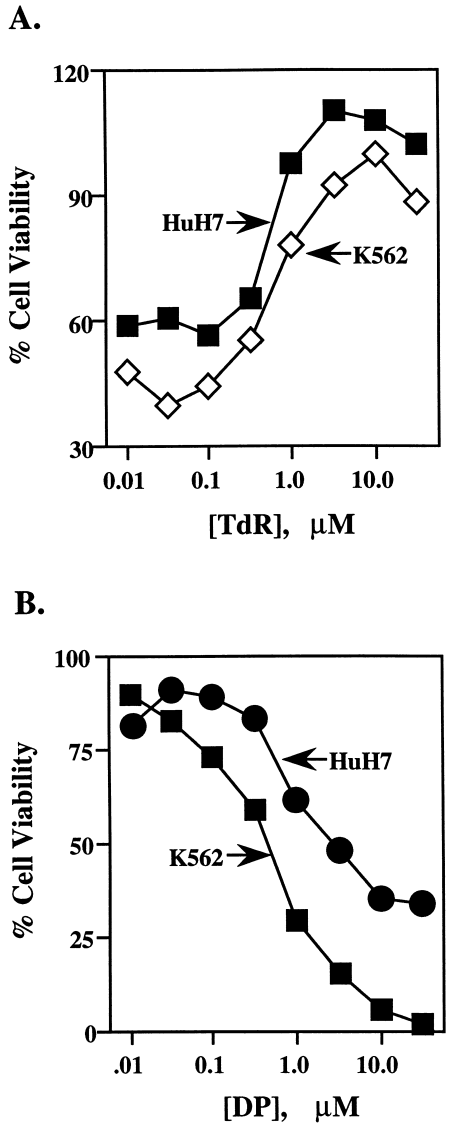

FIG. 6. Nucleoside rescue and its inhibition by nucleoside transport inhibitor in HuH7 and K562 cells. (A) TdR concentration–response relationship. HuH7 and K562 cells were cultured in medium containing 0.3 μM MTX, 100 μM inosine, and various amounts of TdR in a 4-day growth assay. Experiments were performed in triplicate as described in Materials and Methods. (B) DP concentration–response relationship. Cells were cultured in medium containing 0.3 μM MTX, 100 μM inosine, 1.0 μM TdR, and various amounts of DP as indicated on the abscissa. Experiments were performed in triplicate as described in Materials and Methods.

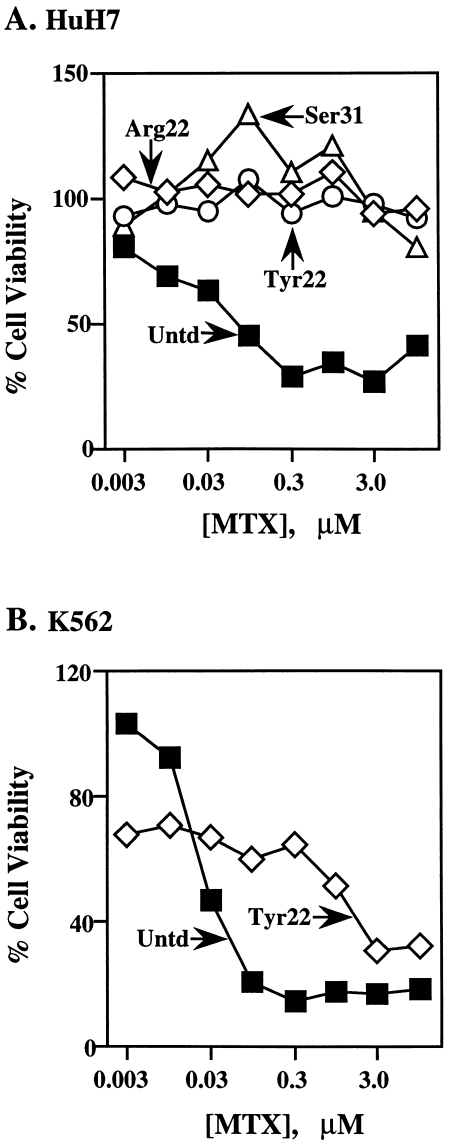

FIG. 7. Effect of nucleoside transport inhibition on MTX concentration–response relationship in HuH7 and K562 cells. Cells were cultured in medium containing 100 μM inosine, 1.0 μM TdR, 5.0 μM DP, and various amounts of MTX. Experiments were performed in triplicate as described in Materials and Methods. Untd, untransduced cells; Arg22, Tyr22, and Ser31 indicate cells transduced by retroviruses encoding variant DHFRs.

tween untransduced and DHFR virus-transduced HuH7 and K562 cells observed in the absence of nucleosides.

DISCUSSION

Conditions were defined under which nucleoside transport inhibition could be used to prevent the rescue of cultured cells from MTX toxicity by the uptake of exogenous nucleotide precursors, thus maintaining selective pressure for cells expressing MTX-resistant DHFR activity. PA317 cells, a murine 3T3 fibroblast derivative, were rescued from MTX toxicity by TdR at concentrations above 1 μM, but this rescue was abrogated by 5 μM DP. Five micromolar DP restored differential MTX toxicity between untransduced PA317 cells and cells transduced with the Arg22, Tyr22, or Ser31 variant murine DHFRs when incubated in the presence of nucleosides. Nucleoside transport inhibition similarly restored differential MTX toxicity between transduced and untransduced HuH7 hepatoma and K562 myeloid leukemia cell lines, implying the usefulness of this approach for a variety of cell types.

Rescue of cells from antifolate toxicity by purine and TdR salvage is well documented, having formed the basis,

for example, of the HAT (hypoxanthine, aminopterin, thymidine) bidirectional selection system for cells expressing functional hypoxanthine-guanine phosphoribosyltransferase and thymidine kinase activities [32]. TdR and inosine also can be used in place of leucovorin for rescue from MTX toxicity in high-dose administration schedules for antitumor chemotherapeutic purposes [33–35]. While salvage pathways can be exploited in certain circumstances to achieve selection or protection from MTX, the use of MTX as a selective agent in combination with drug-resistant DHFRs as a dominant selectable system can be undermined by the same process, due to the presence of undefined components (assumed to be purines and TdR) in various types of sera. This problem can be dealt with effectively in tissue culture systems by the use of medium lacking purines and TdR in combination with either dialyzed serum [2] or serum screened for the absence of such components (McIvor RS, unpublished observation). The use of nucleoside transport inhibition has been investigated as a means to block the uptake of TdR and purines in the nucleoside form. Nelson and Drake [13] found nucleoside transport inhibition to be an effective means of preventing nucleoside rescue from antifolate toxicity in CHO, SAOS-2, and TE-85 cells, demonstrating the ability of DP to sensitize these cell lines to MTX when cultured in medium containing 15% fetal bovine serum. Van Mouwerik *et al.* [36] found a similar enhancement of MTX toxicity by DP in HCT 116 cells cultured in medium supplemented with nondialyzed calf serum.

Rescue from antifolate toxicity is less manageable in the *in vivo* setting, where purines and TdR may be salvageable from the circulation or tissues. Such purine and TdR salvage could blunt the chemotherapeutic effectiveness of antifolates. DP has been tested for its ability to increase the antitumor effectiveness of MTX in Ridgway osteogenic sarcoma tumors in mice, with limited success [13], and in intraperitoneal tumors in humans [18]. In addition, DP has been used in combination with amphotericin B to potentiate the toxicity of MTX in sarcoma 180, cervical carcinoma U14, and Lewis lung carcinoma tumor models in mice [37]. *In vivo* toxicity of trimetrexate (another antifolate) for hematopoietic stem cells has been reported recently to be potentiated by co-administration of the nucleoside transport inhibitor prodrug NBMPR-P [38]. Allay *et al.* [39] have also reported the use of trimetrexate in combination with NBMPR-P to achieve *in vivo* selection of hematopoietic stem cells. These studies indicate, in general, the utility of nucleoside transport inhibition as a means of heightening *in vivo* antifolate toxicity for rapidly dividing cells and improving MTX selectivity against cells not expressing drug-resistant DHFR activity.

Here we have provided a detailed characterization of the conditions under which nucleosides (TdR and inosine, specifically) rescue different cell types from MTX toxicity, and the conditions under which MTX toxicity is restored by nucleoside transport inhibition. These parameters serve to define conditions under which nucleoside transport inhibitors might be applied for improved antitumor efficacy or selectivity of antifolates *in vitro* and *in vivo*, based upon expression of drug-resistant DHFR activity. For example, our results indicated that in the presence of inosine, 1 μM TdR was sufficient to rescue PA317, HuH7, and K562 cells from MTX toxicity, and that under these conditions nucleoside transport inhibition was an effective way of restoring MTX toxicity even in the presence of salvageable nucleoside. Therefore, it may be anticipated that the effectiveness of antifolates may be improved by nucleoside transport inhibition in the mouse, where serum TdR levels have been reported in the 1 μM range [40, 41]. In humans, TdR levels have been reported in the 0.1 μM range [41], a level that provided little to no rescue from MTX toxicity in our experiments. However, since TdR levels in this assay most likely decreased over the duration of the assay, the amount of TdR necessary to rescue cells was probably somewhat lower than 1 μM. Indeed, Radparvar *et al.* [42] found that a constant level of 0.1 μM TdR was sufficient to rescue human colon adenocarcinoma cells from 5′-fluorouracil toxicity in a colony-forming assay. Therefore, it is possible that nucleoside salvage may circumvent antifolate toxicity in humans as well as in mice.

Substantial differences were observed among PA317 (a mouse NIH 3T3 fibroblast derivative), human HuH7 hepatoma, and human K562 myeloid leukemia cell lines in their response to nucleoside rescue from MTX toxicity, and in the maintenance of MTX resistance by expression of different drug-resistant DHFRs. These differences are most likely attributable to variation in the ability of different cell types to transport and metabolize nucleic acid precursors [43–46]. Variation in the expression level of nucleoside transporters, in particular, has been reported for different cell types [47]. Mammalian cells transport exogenous nucleosides through the activity of at least seven different transporters that have been identified. Four have been cloned [48–52] and can be divided into two groups, the Equilibrative Nucleoside Transporters (ENTs) and the Concentrative Nucleoside Transporters (CNTs). The ENTs can be subdivided into equilibrative sensitive (to NBMPR) (*es*) and equilibrative insensitive (to NBMPR) (*ei*). Nucleoside transporters demonstrate substantial species and cell type variations in the levels and types of nucleoside transporters expressed and their interactions with inhibitors [47, 53, 54]. DP, a drug used clinically as a coronary artery vasodilator and as an antithrombotic, inhibits both the *es* and *ei* transport activities in most species.

K562 cells are reported to express both *es* and *ei* nucleoside transport activities [55]. Although the particular nucleoside transport characteristics of the other cell lines used in this study have not been reported, their ability to be rescued from MTX toxicity by TdR and inosine implies the presence of some nucleoside transport activity. Furthermore, this nucleoside rescue was blocked by DP, which inhibits *es* and *ei* transport activities, thus implicating one or both of these transporter activities in the bulk of the rescue observed. HuH7 cells were distinguished from K562

and PA317 cells by their lack of an inosine requirement when rescued by TdR, implicating some other mechanism for satisfying their purine metabolic requirement. In contrast, protection of K562 cells from MTX toxicity by expression of drug-resistant DHFR, either in the absence of nucleosides (Fig. 5) or in the presence of nucleosides plus transport inhibitor (Fig. 7), was incomplete compared with either PA317 or HuH7 cells, consistent with a greater degree of reliance on salvage by some hematopoietic cells for nucleotide biosynthesis [43, 44, 47]. Thus, for cells that efficiently salvage nucleic acid precursors, use of agents that prevent salvage, such as nucleoside transport inhibitors, may be required for maximum therapeutic or selective effectiveness of antifolates.

Another source of variability in the response of different DHFR-transduced cells to MTX is the difference in catalytic activity exhibited by the various DHFRs used in these experiments. As recently reported for transduced K562 cells [4], we observed that the Arg22 DHFR was less effective than the other drug-resistant DHFRs in protecting PA317 cells from MTX at higher levels, most likely due to the extreme reduction in catalytic activity associated with this DHFR variant (about 2% that of WT) in comparison with the Tyr22 (15% of WT) and Ser31 (3–4 times that of WT) murine DHFR variants [30, 31, 56].

Drug-resistant variants of DHFR have been used in several studies to demonstrate protection of animals from antifolate toxicity when such DHFR activity is expressed in all tissues (i.e. in transgenic animals [57, 58]) and specifically in hematopoietic cells, either transplanted from DHFR transgenic donors into syngeneic recipients [5, 9] or by retroviral transduction and transplantation of normal donor marrow into syngeneic recipients [6–8, 10]. Another potential *in vivo* application of drug-resistant DHFR expression is the selective expansion of gene-modified cells by administration of antifolates such as MTX. The ability to selectively expand DHFR-expressing cells could provide a means to increase the *in vivo* representation of such gene-modified cells as a strategy to address the problem of low frequency of gene transfer into a number of different target cell and tissue types [59]. The capability of these different cell types to salvage purines and TdR, thus circumventing the *in vivo* selective pressure of antifolate administration, presents a conundrum for this DHFR-based *in vivo* selective strategy. However, evidence from these *in vitro* studies (this paper) and the *in vivo* studies of Allay *et al.* [38, 39] indicates that this problem can be addressed by coupling antifolate administration with the administration of a nucleoside transport inhibitor, thus allowing selective expansion of DHFR-expressing cells *in vivo* even in the presence of salvageable nucleosides. Animal studies using a DHFR transgenic mouse model system [9, 57] are currently in progress to address this possibility.

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