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Cytotoxic activities of nucleoside and nucleobase analog drugs in malignant mesothelioma: Characterization of a novel nucleobase transport activity☆

Deepti Damaraju[c,d,1], *Vijaya L. Damaraju*[c,d,1], *Miranda Brun*[a], *Delores Mowles*[c,d], *Michelle Kuzma*[c,d], *Richard C. Berendt*[b,e], *Michael B. Sawyer*[c,f], *Carol E. Cass*[a,c,d,*]

[a] *Department of Biochemistry, University of Alberta, Alberta, Canada*
[b] *Department of Laboratory Medicine and Pathology (RCB), University of Alberta, Alberta, Canada*
[c] *Department of Oncology, University of Alberta, Alberta, Canada*
[d] *Department of Experimental Oncology, Cross Cancer Institute, Edmonton, Alberta T6G 1Z2, Canada*
[e] *Department of Laboratory Medicine and Pathology (RCB), Cross Cancer Institute, Edmonton, Alberta T6G 1Z2, Canada*
[f] *Department of Medical Oncology, Cross Cancer Institute, Edmonton, Alberta T6G 1Z2, Canada*

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ABSTRACT

This study was designed to evaluate the cytotoxic activity of several nucleoside and nucleobase analog drugs as possible new agents for treatment of malignant mesothelioma and to identify factors responsible for the clinical variation of nucleoside analog drug response in chemotherapy of mesothelioma. Three human mesothelioma cell lines (MSTO-211H, H2452 and H2052) were tested for gemcitabine sensitivity and nucleoside transport activity. MSTO-211H, H2452 and H2052 exhibited differences in sensitivity to gemcitabine, nucleoside transport rates and hENT1 site densities. In H2052 cells, gemcitabine, 5-fluoro-2′-deoxyuridine, clofarabine and cladribine were most active with IC_{50} values of 46, 43, 240 and 490 nM, respectively, whereas 5-fluorouracil was the least cytotoxic drug tested. In H2052 cells, the combination of gemcitabine and fludarabine or cladribine resulted in synergistic cytotoxic response. In nucleobase transport studies, hypoxanthine and 6-mercaptopurine but not 5-fluorouracil was transported into H2052 cells by a novel purine-specific, sodium-independent nucleobase transport activity. In summary differences in nucleoside analog drug transport activities are likely to contribute to the observed clinical variation in nucleoside analog response in patients and for the first time a correlation between nucleobase drug sensitivities and transport activities was shown. A novel combination of gemcitabine and fludarabine or cladribine had synergistic cytotoxic activity against the least sensitive mesothelioma cell line. These drug combinations merit further evaluation as effective therapeutic regimens in patients with aggressive mesothelioma.

☆ Portions of this work were presented in a preliminary report [1].

* *Corresponding author at*: Department of Oncology, Cross Cancer Institute, 11560 University Avenue, Edmonton, Alberta T6G 1Z2, Canada. Tel.: +1 780 432 8763; fax: +1 780 432 8425.
E-mail address: carol.cass@cancerboard.ab.ca (C.E. Cass).

[1] Both authors contributed equally to this work.

Abbreviations: hENT, human equilibrative nucleoside transporter; NBMPR, nitrobenzylmercaptopurine riboside; IC_{50}, the concentration of drug that inhibited growth of treated cells by 50% relative to that of untreated cells; Cl-dAdo, cladribine; Fara-A, fludarabine; 5-FU, 5-fluorouracil; 6-MP, 6-mercaptopurine; CI, combination index.

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1. Introduction

Malignant mesothelioma is a cancer which arise from surface serosal cells of the pleural, peritoneal, and pericardial cavities. The incidence of this disease is increasing and exposure to asbestos is responsible for 80% of cases [2–4]. Although malignant mesothelioma has a latency period of 20–40 years, it is rapidly fatal once diagnosed with low survival rates (4–12 months) [5,6]. Various chemotherapy drugs, including cisplatin, gemcitabine and antifolate thymidylate synthase (TS) inhibitors, have been assessed either as single agents or in combination [7]. Novel antifolate TS inhibitors (e.g., pemetrexed) are active against a broader range of neoplasms, including lung carcinomas and mesothelioma, compared to classical TS inhibitors [8]. Although a significant survival advantage (12.1 months versus 9.3 months) was demonstrated in recent studies with pemetrexed plus cisplatin versus cisplatin alone, no patients experienced a complete response [6]. Combination chemotherapy with pemetrexed plus cisplatin or gemcitabine plus cisplatin has produced some objective partial responses in the treatment of mesothelioma [6,9].

The goal of the present study was to identify factors that contribute to the differential response in clinic to nucleoside analogs and the underlying transport activities responsible for drug accumulation using three human mesothelioma cell lines, MSTO-211H, H2452 and H2052. We tested the cytotoxic efficacy of several nucleoside and nucleobase analog drugs as possible new agents for treatment of malignant mesothelioma in the cell line least responsive to gemcitabine, and characterized the cellular processes that are involved in the uptake and manifestation of cytotoxicity to these drugs. In addition, we evaluated for the first time a relationship between nucleobase drug sensitivities and underlying transport activites. Many nucleoside analog drugs are DNA damaging agents and induce apoptosis. The capacity of nucleoside analog triphosphates to induce apoptosis through the Apaf-1 mediated pathway demonstrated that cladribine (Cl-dAdo) and fludarabine (Fara-A) were efficient in triggering apoptosis [10]. Combination cytotoxicity studies with gemcitabine and Fara-A or Cl-dAdo in H2052 cells were undertaken based on the mechanistic rationale that both Fara-A and Cl-dAdo are activators of deoxycytidine kinase and Fara-A is a more effective activator of apoptosome than Cl-dAdo or clofarabine [10] and the two drugs will lower the deoxynucleotide pools and inhibit ribonucleotide reductase activity. Gemcitabine is activated by deoxycytidine kinase to its active form and inhibits ribonucleotide reductase and thus combination of purine and pyrimidine antimetabolites may result in enhanced cytotoxic effects against cancer cells.

Efficacy of nucleoside and nucleobase drug therapies is dependent in part on the cellular distribution of various nucleoside and nucleobase transporters. Because pharmacological targets of nucleoside and nucleobase analogs are intracellular, the therapeutic activity of a particular analog is partly determined by the transporters present on tumor cell membranes that deliver the drug intracellularly. Knowledge of the nature of nucleoside and nucleobase transport processes in malignant mesothelioma cells is lacking.

Human equilibrative nucleoside transporters (hENTs) have broad permeant selectivities and wide tissue distributions (for reviews see, [11–13]). hENT1 and hENT2, which are plasma membrane transporters, are functionally distinguished based on their relative sensitivities to nitrobenzylmercaptopurine ribonucleoside (NBMPR) as equilibrative-sensitive and equilibrative-insensitive. Dipyridamole, dilazep and draflazine inhibit both, although hENT2 is much less sensitive than hENT1. Human and rat ENT2 isoforms transport both nucleosides and nucleobases [14]. hENT3 is found in intracellular membranes and thus does not play a role in salvage of external nucleosides [15] and hENT4 is a cell surface pH-dependent transporter with high selectivity for adenosine [16]. A recent study [17] demonstrated that absence of hENT1 in tumors of a proportion of patients with pancreatic cancer who were treated with gemcitabine was associated with decreased survival.

Human concentrative nucleoside transporters (hCNTs) are found primarily in specialized tissues, including intestine, kidney, liver and choroid plexus [18,19]. Three related proteins (hCNT1, hCNT2, hCNT3) have been identified by molecular cloning and functional expression of their cDNAs [20–22]. hCNT1, hCNT2 and hCNT3 mediate, respectively, pyrimidine-nucleoside selective, purine-nucleoside selective and broadly selective nucleoside-transport activities, and hCNT2 also transports uridine [18].

Transport processes for nucleobases have been shown to exist in a range of tissues and cell lines and can be subdivided into equilibrative and concentrative processes [23], but no gene has been cloned encoding the corresponding transporters and knowledge is thus based on functional characterization. Permeation of purine bases in human erythrocytes was shown to occur by a purine-selective sodium-independent process [24]. Although pyrimidine bases, nucleosides and classical inhibitors of nucleoside transport (NBMPR, dilazep and dipyridamole) had no effect, papaverine inhibited purine transport with an IC_{50} value of 5–10 μM [25].

Equilibrative nucleobase transport processes have been identified in human erythrocytes, human T-lymphoblastoid cells, LLC-PK1 cells, rabbit cornea and mouse S49 lymphoma cells [26–29], whereas concentrative nucleoside transport processes have been identified in mammalian kidney, placenta, intestine and choroid plexus [27,30–33]. Sodium-dependent nucleobase transporters are restricted to specialized tissues and are divided into two types: a pyrimidine-selective transporter in the intestine and a more general nucleobase transporter in other tissues [34]. So far absence of nucleobase transport activity was not correlated with lack of drug response.

Since efficacy of nucleoside and nucleobase drug therapies has been linked to underlying transport processes responsible for cellular accumulation of drug in many cell types, we evaluated gemcitabine sensitivity and the underlying transport processes in three mesothelioma cell lines. Site-specific binding studies with NBMPR, a probe for hENT1, identified high-affinity sites on MSTO-211H, H2452 and H2052 cells. In addition, using H2052 cells, we tested several clinically used nucleoside and nucleobase drugs as potential agents for chemotherapy of mesothelioma. Gemcitabine, 5-fluoro-2′-deoxyuridine, clofarabine and Cl-dAdo were most active against H2052 cells with IC_{50} values of 46, 43, 240 and 490 nM, respectively, and hENT1 activity was shown to be

responsible for gemcitabine uptake and no demonstrable activities of hENT2 or hCNTs were observed. In drug combination experiments, a synergistic interaction between gemcitabine and Fara-A or Cl-dAdo was observed. Immunohistochemical studies with monoclonal antibodies against hENT1 in sections obtained from archived tissue samples from three patients with mesothelioma showed positive hENT1 staining thus demonstrating the presence of hENT1 in mesothelioma. In nucleobase transport experiments, hypoxanthine and 6-mercaptopurine (6-MP), but not 5-fluorouracil (5-FU), were shown to be permeants for the purine-specific nucleobase transporter in H2052 cells. Selectivity towards purine nucleobases in uptake experiments in H2052 cells was also seen in cytotoxicity experiments, wherein IC_{50} values for 6-MP and 5-FU were 8.3 ± 1.3 and 330 ± 86 μM, respectively, thus suggesting that membrane permeation is also an important determinant of pharmacological activity of nucleobase drugs in mesothelioma cells.

2. Materials and methods

2.1. Chemicals

NBMPR, dilazep, mineral oil, unlabeled nucleosides, nucleobases, papaverine and other chemicals were obtained from Sigma Chemical Company (Mississauga, ON). Tritiated nucleosides and nucleobases were from Moravek Biochemicals (Brea, CA). Tissue culture (96- and 12-well) plates, cell culture media, and fetal bovine serum (FBS) were from Gibco BRL (Burlington, ON). Ecolite was from ICN Pharmaceuticals (Montreal, PQ) and 550 silicone oil from BC Bearings (Edmonton, AB). The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Kit was from Promega (Madison, WI).

2.2. Cell culture

The human mesothelioma MSTO-211H, H2452 and H2052 cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 10% glucose. All cultures were kept at 37 °C in 5% CO_2/95% air and subcultured every 2–3 days to maintain exponential growth. Transport and cytotoxicity experiments were conducted with cells in exponential growth phase. Cells were free of mycoplasma contamination.

2.3. Cytotoxicity assays

The Cell Titer 96 proliferation assay kit from Promega was used to quantify drug-induced cytotoxicity. Cells were seeded in 96-well plates at 5000 cells/well. After 24 h, cells were exposed to graded concentrations of various cytotoxic purine and pyrimidine nucleoside and nucleobase drugs for 72 h (approximately 3 population doublings) and treated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent for assessment of cytotoxicity. In combination studies drugs were added simultaneously. The concentration of drug that inhibited growth of treated cells by 50% relative to that of untreated cells (IC_{50} values) were calculated from nonlinear regression analyses of data plotted as percentages of control values against the logarithm of drug concentrations using Graph Pad Prism software (San Diego, CA), Version 4.0.

2.4. Analysis of combined drug effects

Drug synergy was determined by the isobologram and combination-index methods, derived from the median-effect principle of Chou and Talalay [35] using the CalcuSyn software (Biosoft, Ferguson, MO). Using data from the MTS cytotoxicity experiments and computerized software, combination index (CI) values were generated over a range of fraction affected (Fa) levels from 0.05 to 0.90 (5–90% growth inhibition). A CI of 1 indicates an additive effect between two agents, whereas a $CI < 1$ or $CI > 1$ indicates synergism or antagonism, respectively.

2.5. Nucleoside and nucleobase uptake assays

Cells were seeded in 12-well plates and after 72 h, rates of uptake of [^{3}H]nucleosides and [^{3}H]nucleobases were measured. For uptake assays, growth media was aspirated and cell monolayers were washed with sodium-containing or sodium-free medium and [^{3}H]permeant (i.e., the appropriate nucleoside or nucleobase) was added and uptake was measured for various time intervals in the presence or absence of potential inhibitors. Transport media consisted of 20 mM Tris, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1.4 mM $CaCl_2$, and 5 mM glucose with either sodium-containing or sodium-free salts at 144 mM and at pH 7.4. At the end of uptake intervals, the permeant was removed and cultures were quickly rinsed twice with either sodium-containing or sodium-free medium after which 5% Triton X-100 was added for solubilization. Each experiment was conducted two or more times in triplicate. Radioactivity in solubilized extracts was measured by liquid scintillation counting. Uptake values were expressed as pmol/10^6cells and graphs were generated using the software GraphPad Prism, Version 4.0.

2.6. NBMPR binding

Binding of [^{3}H]NBMPR was used to quantify the number of hENT1 sites on mesothelioma cells. Cultures in 12-well plates were incubated in triplicate at room temperature for 1 h with 10 nM of [^{3}H]NBMPR in the presence or absence of excess (10 μM) non-radioactive NBMPR in binding medium that consisted of 20 mM Tris, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1.4 mM $CaCl_2$, 150 mM NaCl and 5 mM glucose at pH 7.4. At the end of the incubations, cultures were washed twice with the above buffer, solubilized, and bound [^{3}H]NBMPR was quantified by liquid scintillation counting. Specifically bound [^{3}H]NBMPR was calculated as the difference between the amount of total [^{3}H]NBMPR bound in the absence of 10 μM non-radioactive NBMPR and the amount bound in its presence.

2.7. Immunohistochemistry

Staining was done with tissue samples obtained from three patients. This study was approved by the Research Ethics

Committee of Alberta Cancer Board and patients gave their written informed consent. Immunohistochemistry was performed according to a published protocol [36,37]. Sections (4–6 μm) of formalin-fixed, paraffin-embedded mesothelioma tissue were dried in an oven at 59 °C for 2 h. Sections were rehydrated and, after antigen retrieval and blocking of endogenous peroxidase, were incubated over night at 4 °C with primary anti-hENT1 monoclonal antibodies (monoclonal antibodies specific for hENT1used in this study were developed and characterized previously [38] using synthetic peptides that corresponded to amino acids 254–271) in a humidified chamber for 30 min. Sections were rinsed in phosphate buffered saline (PBS) (pH 7.2), immersed in buffer for 5 min and incubated with DAKO En Vision+ goat anti-mouse dextran conjugate for 30 min. After washing with PBS for 5 min, sections were incubated with diaminobenzidine solution, rinsed and counter stained with hematoxylin. For negative controls, IgG isotype antibodies from mouse were used in the experiments. After washing, stained sections were mounted and imaged using Zeiss Axioskop2 plus Microscope equipped with F Fluar 40×/1.3 oil immersion lens and Zeiss Axiocam color camera (12 megapixels) with 0.63× adaptor.

3. Results

3.1. Gemcitabine cytotoxicity

Cytotoxicity experiments were conducted with gemcitabine, one of the clinically used drugs in treatment of mesothelioma, to determine if the three cell lines MSTO-211H, H2452 and H2052 exhibited differences in sensitivity to gemcitabine. Cells were treated in 96-well plates with graded gemcitabine concentrations (0.0001–100 μM) for 72 h. MSTO-211H, H2452 and H2052 exhibited differential responses to gemcitabine with IC_{50} values (±S.D.) of 3.0 ± 0.7, 43 ± 6 and 49 ± 7, respectively (Table 1).

3.2. Cytotoxicity of nucleoside and nucleobase analog drugs against H2052 cells

Cytotoxicity experiments were conducted with the H2052 mesothelioma cell line (one of two cell lines with lower sensitivity to gemcitabine), to further evaluate the effectiveness of several chemotherapeutic nucleoside and nucleobase drugs as potential therapeutic agents. Of the nucleoside analog drugs tested (Table 2) gemcitabine, 5-fluoro-2′-deoxyuridine, clofarabine and Cl-dAdo were most effective with IC_{50} values (mean ± S.E.) of 46 ± 4, 43 ± 8, 240 ± 40 and 490 ± 60 nM, respectively. Of the nucleobase analogs tested, 6-MP was >40-fold more effective than 5-FU with IC_{50} values of 8.3 ± 1.3 μM and 330 ± 86 μM, respectively.

Table 2 – Effects of cytotoxic nucleosides and nucleobases on H2052 cells

Class	IC_{50} values (μM) ± S.E.
Purine nucleoside analogs	
Clofarabine (Cl-F-ara-A)	0.24 ± 0.04
Cladribine (Cl-dAdo)	0.49 ± 0.06
Fludarabine (F-ara-A)	17 ± 5
Pyrimidine nucleoside analogs	
Gemcitabine (dFdC)	0.046 ± 0.004
Cytarabine (AraC)	1.5 ± 0.2
Thio-Cytarabine (TaraC)	2.7 ± 0.4
5-Fluoro-2′deoxyuridine (5F2′dUrd)	0.043 ± 0.008
5-Fluoro-5′deoxyuridine (5F5′dUrd)	30 ± 10
Nucleobase analogs	
5-Fluorouracil (5-FU)	330 ± 86
6-Mercaptopurine (6-MP)	8.3 ± 1.3

Actively proliferating cultures were exposed to graded concentrations of nucleoside and nucleobase drugs in 96-well plates for 72 h as described in Section 2. IC_{50} values were obtained from the MTS-cytotoxicity relationships and are means (±S.E.) of three experiments, each conducted with six replicates.

3.3. In vitro combination studies with gemcitabine and purine analogs

H2052 cells were seeded in 96-well plates and allowed to attach for 24 h. The cells were exposed to graded concentrations of gemcitabine and purine analogs, either alone or in combination for 72 h. Since the single agent cytotoxicity experiments demonstrated that gemcitabine was more potent than Fara-A (Table 2), combination studies were done at the IC_{50} ratios for gemcitabine with Fara-A, Cl-dAdo or 6-MP over the range of drug concentrations tested. Combination treatments with Fara-A and Cl-dAdo yielded significantly greater cytotoxicity than either agent alone (Fig. 1A and B). Higher synergistic response was observed when gemcitabine and Cl-dAdo were combined at equimolar concentrations (data not shown). The isobologram and combination-index methods developed by Chou and Talalay [35] were used to confirm and quantify the synergism observed with gemcitabine and with Fara-A, Cl-dAdo and 6-MP. Isobolograms were constructed for Fa values of 0.50,

Table 1 – Uptake of 10 μM [^{3}H]uridine, binding of [^{3}H]NBMPR and IC_{50} values for gemcitabine cytotoxicity in three human mesothelioma cell lines

Cell lines	[^{3}H]Uridine (pmol/10^6cells/s)	[^{3}H]NBMPR (Sites/cell) × 10^{-5}	Gemcitabine IC_{50} (nM)
H 211	0.14 ± 0.02	5.8 ± 0.78	3 ± 0.7
H 2452	0.05 ± 0.01	1.9 ± 0.17	43 ± 6.0
H 2052	0.07 ± 0.02	1.9 ± 0.02	49 ± 7.0

Uptake rates (pmol/10^6cells/s) of 10 μM [^{3}H]uridine in three mesothelioma cell lines in the absence or presence of excess non-radioactive uridine, specific binding of [^{3}H]NBMPR (number of hENT1 sites/cell), and gemcitabine IC_{50} values were obtained as described in Section 2. Values shown are means (±S.D.) of three or more experiments, each performed with three or more replicates.

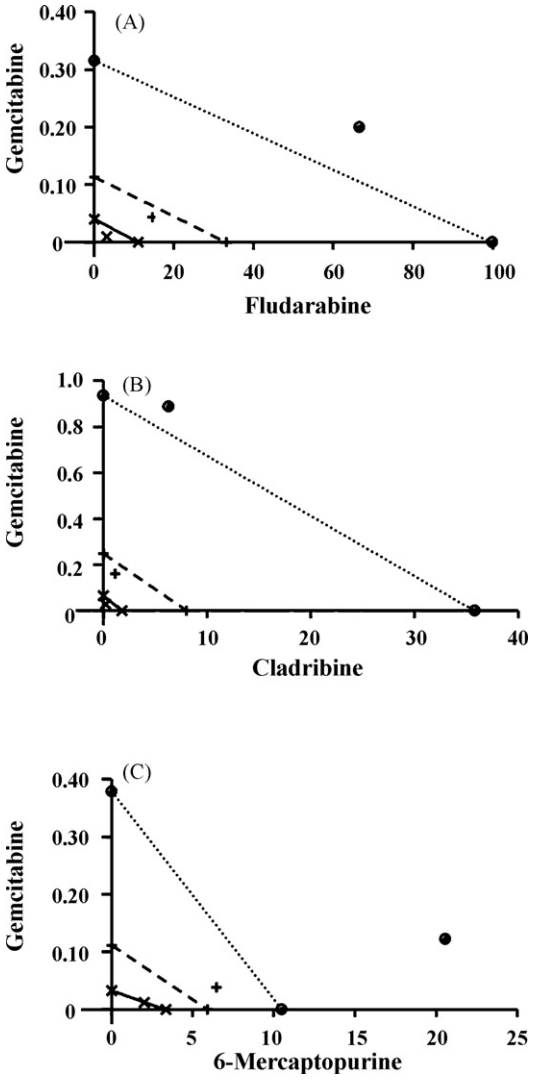

Fig. 1 – Isobolograms of in vitro drug combinations. Isobologram analysis of the combination of gemcitabine and Fara-A or Cl-dAdo or 6-MP in H2052 cells. The individual doses of gemcitabine and Fara-A (panel A) or gemcitabine and Cl-dAdo (panel B) or gemcitabine and 6-MP (panel C) to achieve 90% (dotted line) growth inhibition (Fa = 0.90), 75% (hyphenated line) growth inhibition (Fa = 0.75), and 50% (straight line) growth inhibition (Fa = 0.50) were plotted on the *x*- and *y*-axes. Combination index (CI) values calculated using Calcusyn software is represented by points above (indicate antagonism between drugs) or below the lines (indicate synergy). (X symbol) ED50, (plus sign) ED75 and (open dotted circle) ED90.

0.75, and 0.90, representing 50%, 75% and 90% growth inhibition, respectively (Fig. 1A–C). The CI values at Fa of 0.5 were calculated using CalcuSyn software and these results indicated that gemcitabine and Fara-A or gemcitabine and Cl-dAdo were synergistic with CI values of 0.5, and 0.6, respectively. In contrast combination of gemcitabine and 6-MP was not synergistic (CI value of 1.2).

3.4. [^{3}H]Uridine uptake characteristics

Since gemcitabine cytotoxicity was variable in the three cell lines, uptake of [^{3}H]uridine was evaluated to identify the contribution of nucleoside transport processes in MSTO-211H, H2452 and H2052 cells. Transport of 10 μM [^{3}H]uridine was highest in MSTO-211H cells and rates were lower but similar in H2452 and H2052 cells (Table 1). Fig. 2A shows time courses of 1 μM [^{3}H]uridine uptake (0–90 s) in H2052 cells in sodium-containing and sodium-free media in the presence or absence of 1 mM non-radioactive uridine. Uptake of [^{3}H]uridine was similar in both sodium-containing and sodium-free media indicating the absence of sodium-dependent nucleoside transport activity in H2052 cells. To further delineate the sodium-independent activity in H2052 cells (Fig. 2B), uptake of 10 μM [^{3}H]uridine was monitored up to 90s in the absence or presence of either 0.1 μM NBMPR to inhibit hENT1-mediated transport, or 100 μM dilazep to inhibit both hENT1 and hENT2-mediated components or 1 mM non-radioactive uridine to reveal the passive diffusion component. Results shown in Fig. 1B demonstrate that uptake of [^{3}H]uridine in H2052 cells was mediated by hENT1 since NBMPR, dilazep and non-radioactive uridine yielded similar inhibitions. The presence of hENT1 protein in H2052 membranes was verified by immunoblotting using monoclonal anti-hENT1 antibodies (Fig. 2C). The lower immunoreactive bands in lane C (Fig. 2) could be due to incomplete glycosylation of the hENT1 protein or to proteolysis during storage of membrane fractions. Similar results were obtained in experiments conducted with MSTO-211H and H2452 cells (data not shown).

3.5. Site-specific binding of [^{3}H]NBMPR

Because hENT1 was the only functional nucleoside transporter observed in MSTO-211H, H2452 and H2052 cells, high-affinity interaction of [^{3}H]NBMPR was used to determine hENT1 abundance. MSTO-211H, H2452 and H2052 cells were grown in 12-well plates and incubated with 10 nM [^{3}H]NBMPR with or without 10 μM non-radioactive NBMPR for a minimum of 1 h to ensure that equilibrium between free and bound ligand was reached. Specific binding was calculated as described in Section 2. The binding site densities in MSTO-211H, H2452 and H2052 cells are presented in Table 1. MSTO-211H had the highest number of binding sites and H2452 and H2052 had similar, albeit lower, number of binding sites. These results correlated with observed transport rates and gemcitabine sensitivities in the three cell lines.

3.6. hENT1 immunohistochemistry

We assessed the hENT1 immunohistochemical staining of tissue sections obtained from three patients with malignant mesothelioma to evaluate the presence of hENT1 and to compare the results with the in vitro cell line studies. All three tissues showed positive hENT1 staining on the surfaces of tumor cells (Fig. 3A–C) thus confirming our in vitro observation for the presence of hENT1 in mesothelioma cells. hENT1 staining was not seen when tissues were incubated with IgG isotype control antibodies (Fig. 3D–F) thus showing that the observed staining with the anti-hENT1antibodies was specific.

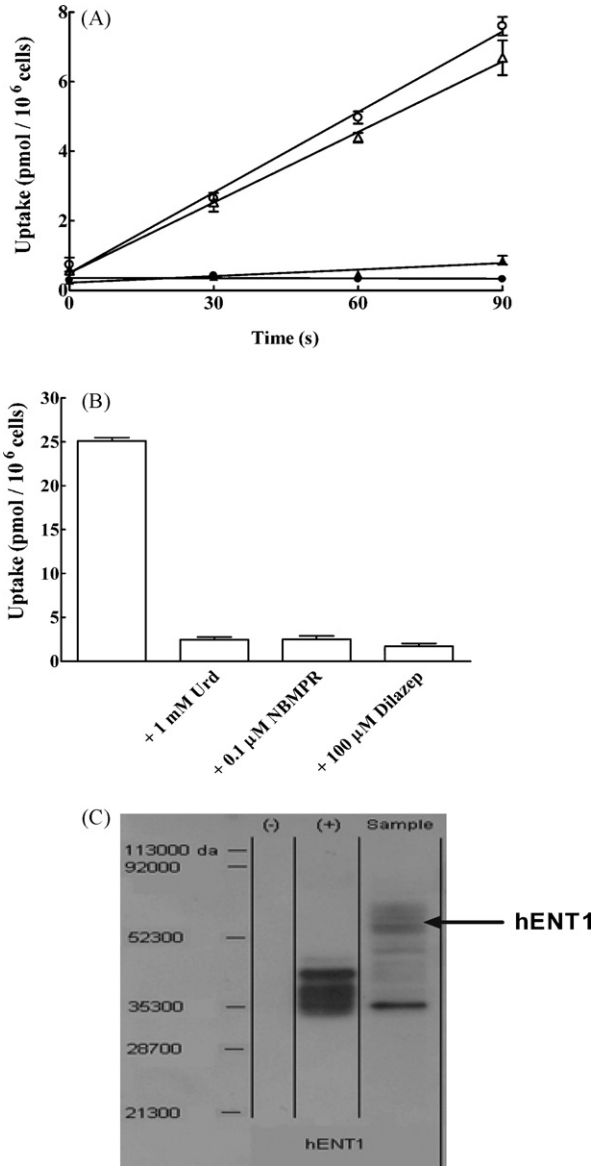

Fig. 2 – Uridine uptake by H2052 cells. Panel A shows H2052 cells that were exposed (from 0 to 90 s) to 1 μM [^{3}H]uridine in sodium-containing medium (○), sodium-free medium (△), sodium-containing medium with 100 μM dilazep (●) and sodium-containing medium with excess (1 mM) non-radioactive uridine (▲). Panel B shows the uptake of 10 μM [^{3}H]uridine at 90 s in the absence or presence of 1 mM non-radioactive uridine, 100 nM NBMPR or 100 μM dilazep. Each bar represents the mean (±S.E.) of two experiments each conducted with three replicates and where the size of the point is larger than the S.E., it is not shown. Panel C shows immunoblotting for hENT1 with membranes from yeast without (−) or with (+) recombinant hENT1 and from H2052 (sample).

Specificity of the anti-hENT1 antibodies was confirmed in peptide competition assays using antibodies preadsorbed with excess immunogenic peptide corresponding to amino acids 254–271 of hENT1 [36,38,39].

3.7. Nucleobase uptake

To assess the role of mediated transport of nucleobase analogs in cytotoxicity against mesothelioma cells a detailed study was undertaken in H2052 cells. Uptake of 10 μM [^{3}H]hypoxanthine was studied in sodium-containing and sodium-free media in the presence or absence of potential inhibitors. Fig. 4A shows the time courses of uptake of 10 μM [^{3}H]hypoxanthine, which were linear and independent of sodium, thus indicating the absence of any sodium-dependent nucleobase transport activity. Inclusion of the nucleoside transport inhibitor dilazep at 100 μM did not inhibit hypoxanthine transport, confirming the lack of involvement of hENT1 or hENT2 in hypoxanthine uptake. Uptake of 10 μM [^{3}H]hypoxanthine was almost completely inhibited by 1 mM excess non-radioactive hypoxanthine, thus showing that hypoxanthine uptake was mediated by a sodium-independent nucleobase transport activity.

In experiments of Fig. 4B, the concentration dependence of hypoxanthine uptake rates was determined to further characterize the hypoxanthine transport activity. Uptake rates exhibited saturation as the concentration was increased from 1 to 1000 μM in sodium-containing medium (Fig. 4B). Kinetic parameters K_m and V_{max} obtained from Michaelis–Menten analysis after correction for the non-saturable component of uptake (mean ± S.E.) were 223 ± 51 μM and 62 ± 5.3 pmol/10^6 cells/s, respectively. These results indicated the presence of a sodium-independent nucleobase transport activity in H2052 cells.

Since 5-FU failed as a chemotherapy agent in treatment of mesothelioma, we tested if 5-FU enters mesothelioma cells by the nucleobase transport activity observed in mesothelioma cells. To assess if 5-FU and 6-MP shared the nucleobase transport activity in H2052 cells, inhibition of 10 μM [^{3}H]hypoxanthine uptake was measured for 1 min or 1 h in the absence or presence of either 1 mM hypoxanthine, 1 mM 6-MP or 1 mM 5-FU. It is evident from Fig. 5 that [^{3}H]hypoxanthine uptake (1 min and 1 h) was strongly inhibited by non-radioactive hypoxanthine and 6-MP, whereas 5-FU had no effect on uptake of [^{3}H]hypoxanthine at 1 min and only a small effect at 1 h, thus indicating that 5-FU was not a good permeant for the nucleobase transport activity observed in H2052 cells.

3.8. Inhibitory effects of nucleosides, nucleobases and transport inhibitors on uptake of [^{3}H]hypoxanthine

Table 3 shows inward fluxes (1 min) of 10 μM [^{3}H]hypoxanthine in the absence (Control, 100%) or presence (% Control) of either (i) 1 mM nucleoside (adenosine, uridine, cytidine or inosine) or nucleobase (hypoxanthine, adenine, guanine, 6-MP, uracil, 5-FU or cytosine), (ii) 10 μM NBMPR and dipyridamole or (iii) 100 μM papaverine, an inhibitor of equilibrative nucleobase transport [25]. While purine nucleobases inhibited uptake of hypoxanthine significantly, pyrimidine nucleobases had very little effect. The nucleobase transport activity observed in H2052 cells was insensitive to nucleosides and classical nucleoside transport inhibitors and was selective towards purine nucleobases.

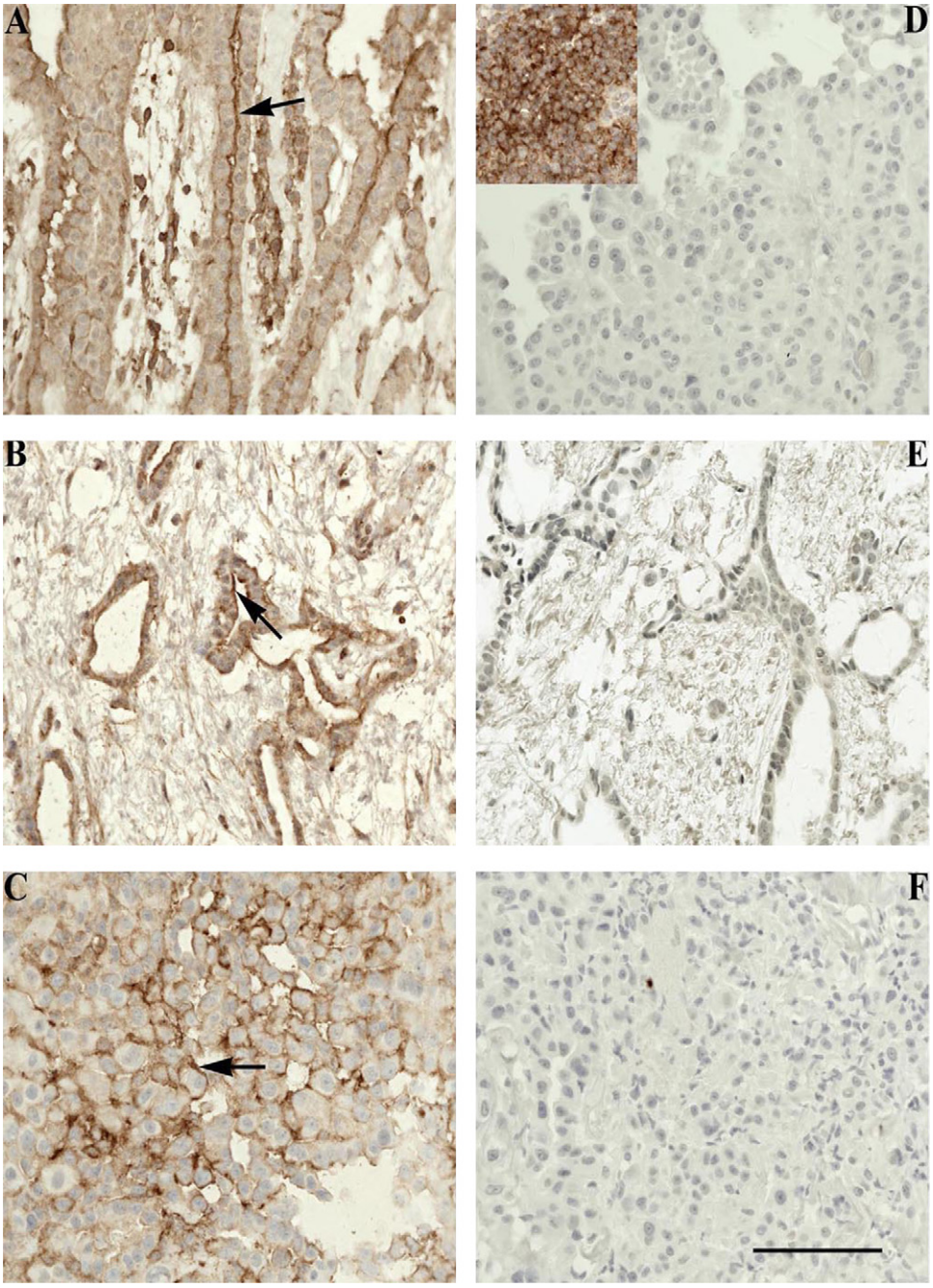

Fig. 3 – Immunohistochemical staining of hENT1 in human mesothelioma tissue sections. Immunohistochemistry was performed on sections derived from formalin-fixed, paraffin-embedded mesothelioma tissue obtained from three patients by incubating with primary anti-hENT1 monoclonal antibodies and IgG isotype control antibodies in a humidified chamber for 30 min. Panels A–C show specific hENT1 staining on the surface of the mesothelial cells; panels D–F show the absence of staining when IgG isotype control antibodies were used in place of anti-hENT1 antibodies. The inset in panel D shows positive staining for hENT1 in lymphocytes of the tumor tissues (internal positive control).

4. Discussion

Nucleoside and nucleobase analogs comprise a family of pharmacologically active cytotoxic drugs used in cancer chemotherapy. Clinically active anticancer pyrimidine and purine nucleoside drugs include cytarabine, gemcitabine, capecitabine, Cl-dAdo, Fara-A and clofarabine [13]. Gemcitabine, a pyrimidine nucleoside analog, is active against solid tumors and some hematological malignancies [40–43]. Some common purine and pyrimidine nucleobase drugs, 6-MP, 6-thioguanine and 5-FU, are cytotoxic and have found therapeutic use as antineoplastic agents [44,45]. Among fluoropyrimidines, 5-FU and capecitabine, a prodrug of 5-FU, were shown to be active in colorectal and breast cancers

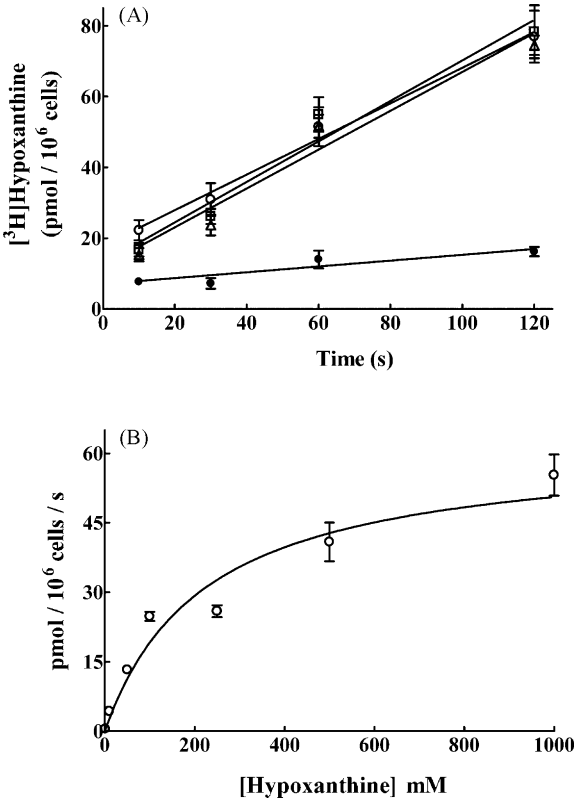

Fig. 4 – Time courses of hypoxanthine uptake by H2052 cells. In panel A, H2052 cells were exposed (from 0 to 120 s) to 10 μM [^{3}H]hypoxanthine in sodium-containing medium (○), sodium-free medium (△), sodium-containing medium with 100 μM dilazep (□) and sodium-containing medium with excess (1 mM) non-radioactive hypoxanthine (●). In panel B, the concentration dependence of hypoxanthine uptake at 30 s is shown. Each data point represents the mean (±S.E.) of three experiments each conducted with three replicates and where S.E. values are not shown, values were smaller than the symbols.

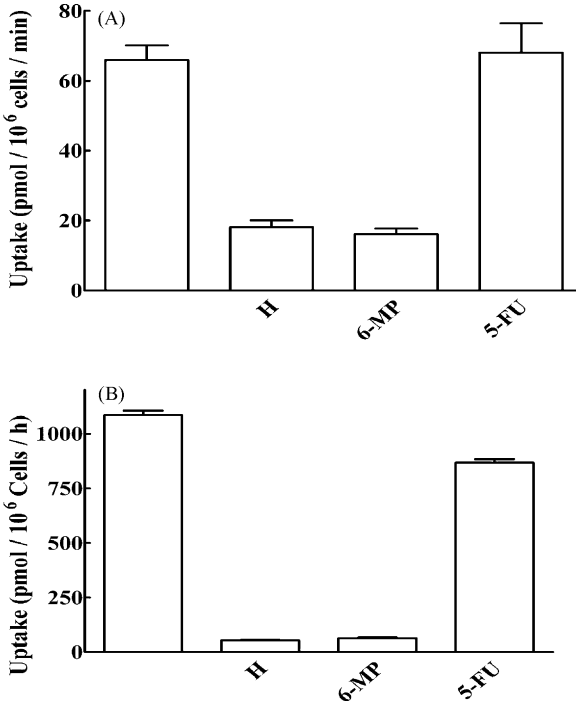

Fig. 5 – Inhibition of hypoxanthine uptake by nucleobases. H2052 cells were exposed (1 min or 1 h) to 10 μM [^{3}H]hypoxanthine in sodium-containing medium with 1 mM non-radioactive hypoxanthine, 1 mM mercaptopurine or 1 mM 5-fluorouracil. Panels A and B show 1 min and 1 h uptake respectively in absence or presence of excess nucleobases. Each data point represents the mean (±S.E.) of three experiments each conducted with three replicates and where S.E. values are not shown, values were smaller than the symbols.

[46,47]. Because pharmacological targets of these drugs are intracellular, the presence of functional nucleoside and nucleobase transporters on cancer cell membranes is an important determinant of their cytotoxicity [17,36].

Pleural malignant mesothelioma is a highly aggressive cancer with a poor prognosis [48] and most patients are potential candidates for chemotherapy at some point during their treatment. With either single agents or combination regimens in phase II trials, rates of tumor regression have been generally under 20% with no significant impact on median survival [49]. Although one study reported a response rate of 48% with combination cisplatin and gemcitabine [9], later studies showed lower efficacy [50]. To understand the variability of response in different clinical trials with cytotoxic nucleoside and nucleobase drugs in mesothelioma, an understanding of processes that contribute to transport of cytotoxic nucleoside and nucleobase drugs is important since variable expression of nucleoside and nucleobase antimetabolite transport activities could lead to unpredictable responses to these agents. In addition, novel combination of nucleoside and nucleobase antimetabolites may lead to enhanced response in resistant phenotypes. Knowledge of the nature of nucleoside and nucleobase transport processes in malignant mesothelioma cells is scarce and a better understanding of the role of various transporters in drug uptake would help in design of more effective chemotherapeutic approaches for mesothelioma treatment.

This study was undertaken to evaluate the in vitro cytotoxic response of three human mesothelial cell lines to gemcitabine, one of the active agents in treatment of mesothelioma, to identify factors responsible for variation of clinical response. Several clinically used nucleoside and nucleobase drugs were tested either alone or in combination against the least sensitive cell line to identify newer cytotoxic agents and to identify the involvement of nucleoside and nucleobase transport activities in uptake of cytotoxic drugs.

In cytotoxicity studies, three cell lines MSTO-211H, H2452 and H2052 were differentially sensitive to gemcitabine with IC_{50} values (±S.D.) of 3.0 ± 0.7, 43 ± 6 and 49 ± 7 nM, respectively. Several chemotherapeutic nucleoside and nucleobase drugs were tested as potential therapeutic agents in H2052 cells (the cell line least sensitive to gemcitabine) and several cytotoxic drugs were shown to be active against H2052 cells in

Table 3 – Effects of nucleosides, nucleobases and nucleoside transport inhibitors on the uptake of 10 μM [^{3}H]hypoxanthine by H2052 cells

Inhibitors	Uptake of [^{3}H]hypoxanthine % control ± S.D.
Nucleosides	
Adenosine	83 ± 4
Inosine	94 ± 11
Uridine	96 ± 7
Cytidine	91 ± 14
Nucleobases	
Hypoxanthine	27 ± 10
Adenine	30 ± 8
Guanine	24 ± 8
6-Mercaptopurine	24 ± 6
Uracil	91 ± 12
5-Fluorouracil	103 ± 32
Cytosine	91 ± 7
Inhibitors	
NBMPR	105 ± 5
Dipyridamole	98 ± 11
Papaverine	27 ± 10

Uptake of 10 μM [^{3}H]hypoxanthine for 1 min was determined in the absence (100%) and presence (% Control) of 1 mM of nucleosides or nucleobases, 10 μM NBMPR or dipyridamole or 100 μM papaverine. [^{3}H]Hypoxanthine and individual test compounds were added simultaneously. Values shown are means (±S.D.) of three (inhibitors) or four experiments (nucleosides and nucleobases), each performed in triplicate.

addition to gemcitabine. 6-MP, Fara-A, Cl-dAdo (purine-like) and gemcitabine, (pyrimidine-like) antimetabolites inhibit purine and pyrimidine nucleotide synthesis pathways in cells, leading to inhibition of cellular enzymes responsible for maintenance of dNTP pools and resulting in inhibition of DNA and RNA synthesis. Combination of purine and pyrimidine antimetabolites may result in enhanced cytotoxic effects against cancer cells. In combination cytotoxicity studies using gemcitabine and purine analog drugs, the combination of gemcitabine and Fara-A was synergistic in H2052 cells and the dose of gemcitabine could be reduced by >10-fold and Fara-A by ~5-fold when used in combination. The combination of gemcitabine and Cl-dAdo was more synergistic when the two drugs were combined at equimolar ratios than at their IC_{50} ratios. However the combination of gemcitabine and 6-MP was not synergistic in H2052 cells. Our data suggest a synergistic interaction between gemcitabine and Fara-A or Cl-dAdo at pharmacokinetic concentrations and further experiments are needed to understand the basis of this synergy and to explore other combination drug treatments.

Uptake of uridine, a universal permeant for all known human nucleoside transporters, and NBMPR binding were measured in all three cell lines. In uridine uptake experiments the three cell lines exhibited different rates of uridine transport with MSTO-211H exhibiting the highest uptake rates. Binding studies with [^{3}H]NBMPR in the three cell lines correlated with the uptake rates and gemcitabine sensitivities. Based on these results, nucleoside drug transport in the three cell lines was primarily mediated via hENT1. For example, hENT1 was the major route of gemcitabine entry into the three cell lines as reflected by the differences in gemcitabine sensitivity as a function of hENT1 site densities. Since information about the nucleoside transport processes in malignant mesothelioma is lacking, using [^{3}H]uridine as a permeant, a detailed analysis of nucleoside transport processes was undertaken in all three cell lines and representative results with H2052 cells were shown. Using inhibitors of equilibrative nucleoside transport processes and sodium-containing and sodium-free media, all three cell lines were shown to exhibit only hENT1 activity. NBMPR-sensitive hENT1 activity was responsible for the transport of both purine and pyrimidine nucleoside drugs.

In hENT1 immunohistochemical staining studies positive staining for hENT1 was observed in tissue sections of three patients with malignant mesothelioma, consistent with the observation of the presence of hENT1 in the three mesothelioma cell lines. These studies form the basis for future work, wherein gemcitabine drug treatment response could be correlated with expression levels of hENT1.

To explore the causes for failure of 5-FU in treatment of mesothelioma we studied the 5-FU transport activity in H2052 cells. 5-FU was a poor permeant for the nucleobase transport activity in H2052 cells similar to the erythrocyte nucleobase transport activity observed previously (K_m for hypoxanthine and uracil were 180 ± 12 and 5800 ± 700 μM, respectively) [24]. These results suggest that a commonly used chemotherapeutic nucleobase 5-FU was not a permeant for the nucleobase transport activity observed in H2052 cells and may explain in part why 5-FU failed as an agent in treatment of mesothelioma. Thus, cytotoxicity and transport studies with gemcitabine and 5-FU in H2052 cells provided evidence that transportability was an important determinant for the pharmacological activity of nucleoside and nucleobase drugs.

Functional nucleobase transport activity has not been identified to date in malignant mesothelioma; in this study, the presence of a nucleobase transport activity in H2052 cell line was evaluated using [^{3}H]hypoxanthine as a permeant. Although, hENT2 can transport bases, H2052 lacked this transport activity. Our studies identified the presence of a novel sodium-independent nucleobase transport activity that was insensitive to classical nucleoside inhibitors NBMPR, dilazep, dipyridamole and resembles the nucleobase transport activity of erythrocytes [24]. Transport of hypoxanthine was saturable with K_m and V_{max} values of 223 ± 51 μM and 62 ± 5 pmol/10^6 cells/s, respectively. H2052 cells exhibited hypoxanthine, 6-MP and adenine transport activity whereas their capacity to transport 5-FU was weak. [^{3}H]Hypoxanthine uptake by H2052 cells was inhibited by purine nucleobases (adenine, guanine, 6-MP and hypoxanthine) but not by pyrimidine nucleobases (uracil, 5-FU and cytosine). The uptake was also sensitive to papaverine, consistent with the presence of a purine-selective sodium-independent nucleobase transport activity in H2052 cells.

In summary our results identified differences in gemcitabine sensitivities, nucleoside transport rates and hENT1 site densities among three mesothelioma cell lines. We also demonstrated hENT1-mediated nucleoside transport activity and a novel sodium-independent purine-selective nucleobase transport activity in the three cell lines that contributed to antimetabolite cytotoxicity and identified clofarabine, Cl-dAdo,

gemcitabine, 5-fluoro-2′-deoxyuridine and 6-MP as potent nucleoside and nucleobase drugs active against the least sensitive H2052 mesothelioma cell line. In combination cytotoxicity studies synergy with gemcitabine and Fara-A or Cl-dAdo was observed with H2052 cell line. The failure of 5-FU in treatment of mesothelioma may have been due to lack of transport activity for this drug. Based on these results we conclude that variations in transportability could manifest as differences in cytotoxicity/response. We believe that selective use of antimetabolites based on mechanistic rationales could lead to improved treatment outcomes and possibly minimize side-effects in a clinical setting and recommend that the combination of gemcitabine and Fara-A or Cl-dAdo merits further evaluation as an effective therapeutic regimen in patients with aggressive mesothelioma.

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