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Hypoxanthine transport in human tumour cell lines. Relationship to the inhibition of hypoxanthine rescue by dipyridamole

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Abstract

Hypoxanthine (HPX) uptake was investigated in four human tumour cell lines previously characterised as being sensitive (*ds*: A549 and MCF7) or insensitive (*di*: COR-L23 and T-47D) to dipyridamole (DP)-induced inhibition of HPX rescue from antipurine antifolate-induced growth inhibition. The aim of the study was to determine the mechanism underlying the differential sensitivity of HPX rescue to DP. The time–course of HPX uptake in the two *ds* cell lines was different in comparison to the two *di* cell lines. The initial rate of HPX uptake in the *di* cell lines was more rapid than in the *ds* cell lines such that at 60 sec the amount of HPX taken up by the former was 2–6 times higher than that taken up by the later. The K_t and T_{max} for HPX transport in *di* COR-L23 cells were 870 μM and 4.75 μM/10^6 cells/min and 1390 μM and 1.78 μM/10^6 cells/min in *ds* A549 cells. HPX transport was not sodium-dependent in these cells. Equilibrative nucleoside transporter 2 (ENT2)-mediated thymidine transport was also higher in *di* cells. DP inhibited HPX uptake into *ds* cell lines by $\geq$48% and by $\leq$20% in the *di* cell lines. Competition studies with HPX and thymidine transport via ENT2 indicated an overlap between nucleoside and nucleobase transport transporters in the breast cancer cell lines (MCF7 and T-47D). These studies showed that more rapid and extensive HPX uptake, as well as reduced sensitivity to DP inhibition, is associated with the inability of DP to prevent HPX rescue from antipurine antifolate-induced growth inhibition in certain human tumour cell lines.

Keywords: Nucleobase transport/inhibition; Hypoxanthine transporters; Human tumour cells

1. Introduction

Nucleoside and base transport is an important factor during antimetabolite cancer chemotherapy either because the antimetabolites are nucleosides or bases, and hence substrates for transport, or because they inhibit *de novo* nucleotide biosynthesis and hence stimulate salvage.

Nucleosides and bases enter cells by carrier-mediated processes that are either equilibrative or concentrative in nature. Two genetically distinct equilibrative nucleoside transporters have been identified that differ in their kinetic properties and sensitivities to low concentrations of NBTI. The equilibrative NBTI-sensitive transporters, termed *es*, are inhibited by low nanomolar concentrations of NBTI, whereas the equilibrative NBTI-insensitive transporters, termed *ei*, are unaffected by 1 μM NBTI [1,2]. The *es* and *ei* transporters have recently been cloned and the proteins have been designated ENT1 and ENT2, respectively [3–5]. In addition to equilibrative transporters, at least five sodium-dependent CNT processes have been identified, and two of these transporter types have been cloned [6–9]. Different types of CNTs vary in their permeant selectivities, inhibitor sensitivity, and distribution [6–9].

In contrast to nucleoside transport, nucleobase transport has been less extensively studied. In a number of mammalian cell lines classed as Group I cells by Plagemann *et al*. [10] (e.g. Chinese hamster lung and ovarian cells and Novikoff rat hepatoma cells), influx of 500 μM HPX was inhibited by DP and uridine, but not NBTI [10,11]. These cell types have been found to possess relatively high proportions of ENT2 (30–50%), suggesting that HPX transport was occurring via ENT2. Evidence for nucleobase transport

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Abbreviations: ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter; DP, dipyridamole; HPX, hypoxanthine; NBTI, nitrobenzylthioinosine; TdR, thymidine; K_t, kinetic constant for tansport; and T_{max}, maximum velocity for transport.

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via ENT1 is restricted to mouse S49 lymphoma cells, where the K_m was relatively high (~1 mM) [12].

HPX uptake in other cell types, classified as Group II cells (e.g. human erythrocytes and L929 and P388 mouse leukaemia cell lines), was insensitive to inhibition by DP and uridine, indicating the existence of specific nucleobase transporters [10]. Human erythrocytes and pig renal epithelial LLC-PK_1 cells have been shown to possess facilitated diffusion-mediated transporters with a relatively high affinity for purine bases (apparent K_m for HPX 18 μM and 12 μM, respectively), and a low affinity for pynmidine bases [13–16].

In addition to high-affinity equilibrative purine transporters, LLC-PK_1 cells have also been shown to possess CNTs, which transport purine and pyrimidine bases with high affinity (apparent K_m values ~10 μM) and are inhibited by DP (apparent K_i value of 4.6 μM) [16]. Sodium-dependent nucleobase transport has also been demonstrated injejunal rat tissue and opossum kidney epithelial cells [17,18].

Exogenous HPX can circumvent the growth-inhibitory properties of the antipurine antifolates, e.g. lometrexol, LY309887, and AG2034 [19–21]. Recently, studies have demonstrated that the effect of DP on HPX-mediated rescue from antipurine antifolate-induced growth inhibition may be selective for tumour cells and not sensitive normal tissues [22,23]. DP prevented HPX rescue from lometrexol-induced growth inhibition in some tumour cell lines, resulting in a decrease in cell number (termed DP-sensitive or *ds* cells). In other cell lines and in primary cultures of haematopoietic and intestinal cells, growth continued at the same rate as that of untreated control cells (termed DP-insensitive or *di* cells) [22,23].

In order to determine the potential for selective anticancer therapy using antimetabolite–DP combinations, we undertook the study reported here, namely: to investigate the mechanisms underlying the sensitivity of HPX rescue to DP. In this, the first study of HPX uptake in human tumour cell lines, the differential sensitivity of HPX transport to DP was studied in cell lines previously characterised as having DP-sensitive (*ds*) or DP-insensitive (*di*) HPX rescue [23]. More rapid and extensive HPX uptake, as well as reduced sensitivity to DP inhibition, was associated with the inability of DP to prevent HPX rescue from antipurine antifolate-induced growth inhibition in the human tumour cell lines studied.

2. Materials and methods

2.1. Materials

Foetal bovine serum was purchased from Globepharm. Dulbecco's A phosphate-buffered saline, penicillin, and streptomycin were obtained from GIBCO. RPMI-1640 medium, DP, NBTI, TdR, HPX, and all other reagents were purchased from Sigma Chemical Co. [*Methyl*-^{3}H]TdR (final specific activity 92.5 GBq/mmol), [U-^{14}C]sucrose (final specific activity 7.4 GBq/mmol), and [8-^{3}H]HPX (final specific activity 39 GBq/mmol) were purchased from Amersham International.

2.2. Cell culture

MCF7 and T-47D breast adenocarcinoma cells were obtained from the European Collection of Animal Cell Cultures and had doubling times of 33 and 37 hr. respectively. COR-L23 (doubling time = 32 hr) and A549 non-small cell lung carcinoma (doubling time = 24 hr) were obtained from Dr. Peter Twentyman (MRC Clinical Oncology and Radiotherapeutics Unit, Cambridge, Cambs., UK) and the National Cancer Institute, respectively. Cells were grown in RPMI-1640 medium supplemented with 1000 units/mL of penicillin, 100 μg/mL of streptomycin, and 10% (v/v) foetal bovine serum at 37° in a 5% CO_2-enriched humidified atmosphere. A Hoechst 33258 DNA fluorescence technique [24] was used monthly to ensure that all cell lines were mycoplasma-free.

2.3. Thymidine transport assay

Zero-*trans* uptake of TdR was measured in A549, COR-L23, MCF7, and T-47D cells using a method adapted from the rapid transport assay [25] and the inhibitor stop technique [13,26]. Cells were harvested using Dulbecco's A phosphate-buffered saline containing 400 mg/L of EDTA and were transferred to 50-mL centrifuge vials and centrifuged at 1850 *g* for 5 min at 4° in a Mistral 3000 centrifuge. The supernatant was aspirated and the cells were resuspended in 50 mL ice-cold transport buffer containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 10 mM glucose, and 10 mM HEPES adjusted to pH 7.4. Cells were centrifuged at 1850 *g* for 5 min at 4°, the supernatant was removed, and the cells were resuspended at 1–2 $\times$ 10^7 cells /mL in transport buffer and left on ice to recover for 30 min.

Cells were preincubated with NBTI or DP, dissolved in DMSO, or DMSO alone for 5 min. at 21°. One hundred microliters of cell suspension (containing approximately 1 $\times$ 10^6 cells) was pipetted into 0.4-ml microcentrifuge tubes (BDH) containing a layer of 150 μL Dow Corning silicone oil (final specific gravity 1.028) (45 mL Dow Corning 556 silicone oil [specific gravity 0.98] + 55 mL Dow Corning 550 silicone oil [specific gravity 1.068]) which had been previously placed over 50 μL 3M KOH. Transport was initiated by adding 50 μL of TdR in transport buffer containing [*methyl*-^{3}H]TdR and [U-^{14}C]sucrose, at the final specific activities given above, to each tube at 2-sec intervals using a multipette (Eppendorf) and a metronome to keep time, giving final concentrations of 10^6 cells in 150 μL transport buffer, with 100 μM or 200 μM TdR and 5% (v/v) DMSO ± DP and/or NBTI. In competition experiments, inosine or HPX was also added to the transport buffer to

give a final assay concentration of 200 μM. Transport was stopped by waiting 1 sec after the final addition before adding 50 μL of 400 μM DP in transport buffer at 2-sec intervals to each tube in reverse order. To transfer the cells into the KOH layer, tubes were placed in an Eppendorf 5415C centrifuge and centrifuged at 6700 *g* for 2 min at room temperature. Each assay was carried out in triplicate, and the cell number and viability was determined with a haemocytometer chamber using the trypan blue exclusion method.

After centrifugation, the cells which had passed through the oil layer were left to lyse in the KOH for 1 hr. Tubes were then cut through the oil layer and the bottom of the tube allowed to fall into scintillation vials (North Eastern Laboratory Supplies). One millitre of 0.25 M acetic acid was squirted into the tube bottoms to neutralise the KOH and reduce chemoluminescence during liquid scintillation counting on a Wallac 1410 liquid scintillation counter (Pharmacia Wallac) for 1 min per sample. Standards were prepared by adding 10 μL[^{3}H]TdR–[^{14}C]sucrose radiolabel mix to 10 mL scintillation fluid. These standards were used to calculate the [^{14}C]:[^{3}H] ratio and hence to determine the amount of non-transported [^{3}H]TdR in the extracellular space that was carried through the oil layer.

2.4. Hypoxanthine transport assays

HPX transport was measured in a similar way to that described for TdR transport; however, an oil-stop method was used instead of an inhibitor-stop method and the transport buffer contained 140 mM sodium chloride and 10 mM HEPES pH 7.4 One million cells preincubated with NBTI, DP, or DMSO control were layered over oil, and transport was initiated by adding 50 μL of transport buffer containing HPX at various concentrations and [8-^{3}H]HPX and [U-^{14}C]sucrose to each tube. In competition experiments, inosine or TdR was also added to the transport buffer. For the measurement of HPX uptake at 1-sec intervals, a multipette and a metronome were used as described above. HPX uptake measurements over 1-min intervals were conducted using a digital timer. Transport was stopped by centrifuging the tubes at 6700 *g* for 2 min to pellet the cells into the KOH layer. Each determination was carried out in triplicate. Cell number and viability was determined with a haemocytometer chamber using the trypan blue exclusion method, and the tubes were subsequently processed for scintillation counting as described above. When the absence of sodium was required in an experiment, 140 mM potassium chloride was substituted for sodium chloride in the transport buffer.

K_i values for inhibition of HPX uptake by DP in A549 and COR-L23 cells were calculated using the equation:

$$K_i = \frac{K_t[I]}{K_{ti} - K_t}$$

where K_t is the HPX transport constant in the absence of inhibitor, [I] is the concentration of inhibitor (10 μM), and K_{ti} is the transport constant in the presence of 10 μM DP.

2.5. High performance liquid chromatography

To investigate the extent of HPX metabolism under the conditions of the uptake assay, the intracellular fate of HPX was determined by HPLC. Cells were harvested and prepared as described for the HPX transport studies. Cell suspensions were then incubated for 5 min at 21° and then pipetted into microfuge tubes containing a layer of 150 μL Dow Corning silicone oil overlaying 100 μL 1 M cold perchloric acid (PCA).

Transport was initiated by adding 50 μL of HPX radiolabel and the tube was placed in an Eppendorf 5415C centrifuge; after 60 sec, transport was stopped by centrifuging the tubes at 6700 *g* for 2 min at room temperature. Each run was carried out in triplicate, and cell number and viability was determined with a haemocytometer chamber using the trypan blue exclusion method.

After 1 hr at 4° to allow for PCA precipitation of macromolecules, the bottoms of the microfuge tubes were then cut off and allowed to fall into Eppendorf tubes. One hundred microlitres of deionised water was added to each Eppendorf tube to dilute the PCA. The liquid contents of the tube were transferred to another Eppendorf tube, which was then centrifuged at 6700 *g* for 2 min at room temperature to pellet the precipitated macromolecules. The resulting supernatant was neutralised with sodium bicarbonate and stored at −20° prior to HPLC analysis. The radioactivity in the PCA pellet was determined to ensure that radiolabelled HPX was not being incorporated into RNA and DNA during the assay period.

[^{3}H]HPX-derived material from the supernatant was resolved on a 150-mm Nucleosil 3 μ ODS cartridge (Jones Chromatography) using an Alliance Waters 2690 separation module (Waters) with a mobile phase consisting of 0.02 M sodium acetate pH 4, pumped isocratically at 1 mL/min for 10–12 min. Samples were analysed using an on-line FLO-ONE radiochromatography detector (Packard) with a liquid scintillant cell (flow rate of liquid scintillant [Packard] of 2 mL/min). UV absorbance of standard and endogenous HPX, AMP, GMP, and IMP was measured on a Waters 960 photodiode array detector operating over a wavelength of 210–240 nm. UV chromatograms were collected using Millenium 3.05 Client Server software (Waters), and the radiochromatograms were collected initially using the FLO-ONE data system (Packard) with analogue output to the Millenium software.

2.6. Data analysis

Initial linear rates of HPX and TdR uptake and K_t and T_{max} values for HPX uptake were calculated using unweighted linear regression analyses (GraphPad Prism Soft-

ware). Statistical analyses of data by unpaired, two-tailed Students' *t*-test were performed using Instat2 (GraphPad Prism Software).

3. Results

3.1. Determination of ENT2-mediated TdR transport in human tumour cell lines

To investigate if there is a relationship between the extent of ENT2-mediated TdR transport and the sensitivity of HPX rescue to DP, the uptake of 100 μM TdR over 12 sec was measured in the *ds* A549 and MCF7 cell lines, and in the *di* COR-L23 and T-47D cell lines, in the presence of 0.5–15,000 nM NBTI. A plateau in the inhibition of the TdR uptake rate was seen between 5 and 500 nM NBTI for COR-L23, MCF7, and T-47D, and between 5 and 50 nM NBTI for A549 cells (data not shown). This biphasic profile is indicative of the cell lines possessing both ENT1 and 2 as previously described [2]. The results presented here indicate that rates of ENT2-mediated TdR transport were higher in *di* COR-L23 and T-47D cell lines (Table 1). However, there was no obvious relationship between the percentage of ENT2-mediated TdR uptake in the four cell lines and the sensitivity of HPX rescue to DP.

Table 1
Rates of ENT1- and ENT2- mediated TdR transport in human tumour cell lines

Cell line	Rate of TdR uptake via ENT1 (pmol/10^6 cells/sec)	Rate of TdR uptake via ENT2 (pmol/10^6 cells/sec) [% of total TdR uptake]
A549	4.9 ± 0.2	1.6 ± 0.3 [25 ± 6]
COR-L23	8.7 ± 0.1	3.9 ± 0.4 [31 ± 3]
MCF7	5.3 ± 0.8	2.3 ± 0.5 [31 ± 7]
T-47D	3.2 ± 0.9	5.1 ± 0.6 [63 ± 11]

Rates of control [^{3}H]TdR uptake remaining in the presence of 50 nM NBTI was measured as described in Materials and Methods. Results are means ± SD of three experiments.

3.2. HPX uptake

Uptake of 200 μM HPX was measured over short (7–10 sec) and long (1–4 min) time–courses using the oil-stop method in the four cell lines (Fig. 1). In untreated cells, the initial rates of transport were linear over 10 sec and were 4.8 ± 3 and 10 ± 2 pmol/10^6 cells/sec for A549 and MCF7 cell lines with *ds* HPX rescue, and 83 ± 14 and 23 ± 6 pmol/10^6 cells/sec for the *di* COR-L23 and T-47D cell lines. These results indicate that the rate of initial uptake of HPX is higher in the cell lines with DP-insensitive HPX rescue, particularly so in COR-L23 cells. Over the longer

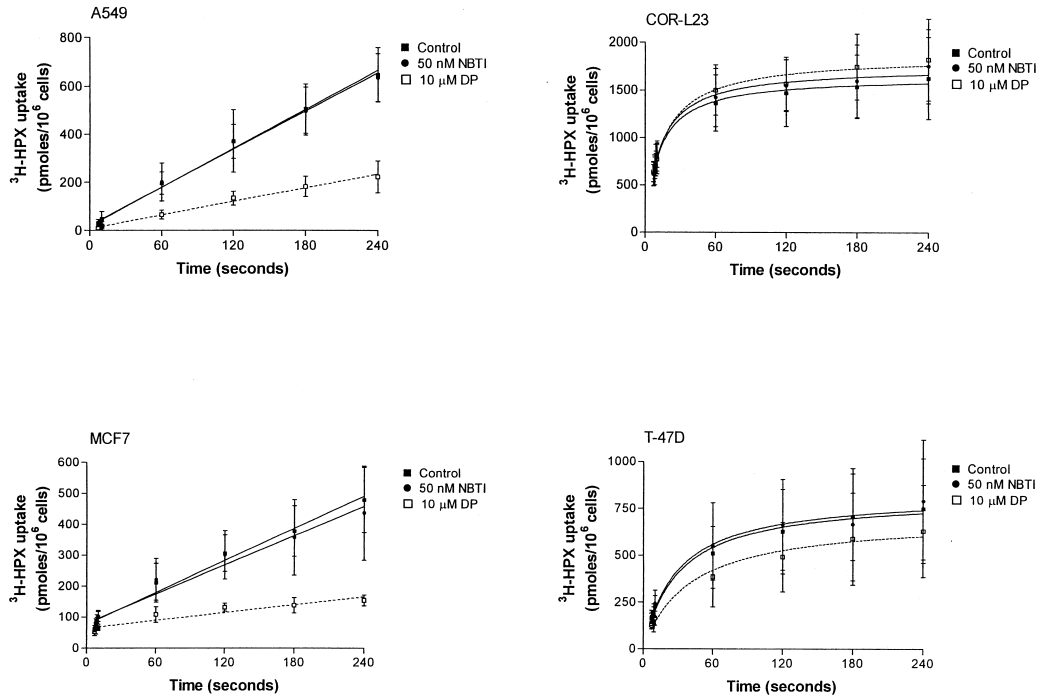

Fig. 1. Time–course of HPX uptake in four human tumour cell lines. A549, MCF7, COR-L23, and T-47D cells were incubated in DMSO (control), ■; 50 nM NBTI, ●; or 10 μM DP, □ for 5 min before uptake was initiated with 200 μM [^{3}H]HPX in transport buffer and stopped at the appropriate time by centrifugation through oil. Cells were lysed with 3 M KOH and cell-associated radioactivity was determined. Lines and curves were fitted as described in Methods and points are the mean ± SD of three experiments.

Table 2
Effect of DP on [^{3}H]HPX uptake in four human tumour cell lines

Cell line	HPX uptake at 60 sec (pmol/10^6 cells)	HPX uptake at 60 sec + 10 μM DP (pmol/10^6 cells)
A549	200 ± 50	66 ± 20*
COR-L23	1370 ± 300	1500 ± 260
MCF7	210 ± 60	110 ± 30*
T-47D	510 ± 140	390 ± 160*

Uptake of [^{3}H]HPX after 60 sec was measured as described in Materials and Methods. Data are means ± SD for three determinations.

* Significantly different from uptake in the absence of DP ($DP < 0.005$ in unpaired Student's *t*-test).

time–course of 60 sec, these differences in the uptake of HPX by the cell lines were maintained, as shown in Table 2.

As indicated in Fig. 1, the characteristics of HPX uptake over time were different in the cell lines. In the *ds* cell lines (A549 and MCF7), HPX levels increased linearly with time; however, in the *di* cell lines (COR-L23 and T47D), HPX uptake was very rapid within the first 60 sec, reaching a plateau thereafter. At 60 sec, the *di* cells had accumulated 2–6 times as much HPX as the *ds* cells

3.3. Inhibition of HPX uptake by DP and NBTI

As expected on basis of the growth inhibition data [23], DP significantly inhibited HPX uptake in the two *ds* cell lines, A549 and MCF7, by 67 and 48% ($p < 0.0001$), respectively. No inhibition of HPX uptake by DP was observed in COR-L23 cells, which have *di* HPX rescue; however, DP significantly reduced HPX uptake by 24% in the T-47D *di* cell line ($p < 0.01$). In all the cell lines studied, NBTI did not significantly affect HPX transport, which indicates that HPX transport is not mediated by ENT1 or any other NBTI-sensitive transporter in any of these cell lines (Fig. 1).

3.4. Determination of kinetic and inhibition constants for HPX transport and inhibition in A549 and COR-L23 cells

The uptake of HPX after 60 sec over a wide range of HPX (25–800 μM) concentrations was measured in COR-L23 and A549 cells in the presence and absence of 10 μM DP (Fig. 2). The K_t values for HPX uptake into A549 and COR-L23 cells were 1390 ± 510 μM and 870 ± 230 μM, respectively, with T_{max} values of 1.78 ± 0.46 μmol/10^6 cells/60 sec and 4.75 ± 0.77 μmol/10^6 cells/60 sec, respectively. These values are higher than those determined previously for HPX influx into human erythrocytes and LLC-PK$_1$ porcine renal epithelial cell line (K_m = 120 and 124 μM, respectively), yet not as high as the constants calculated for HPX influx via the ENT2 in Chinese hamster lung and ovary cells (K_m = 2.0 and 2.5 mM, respectively)

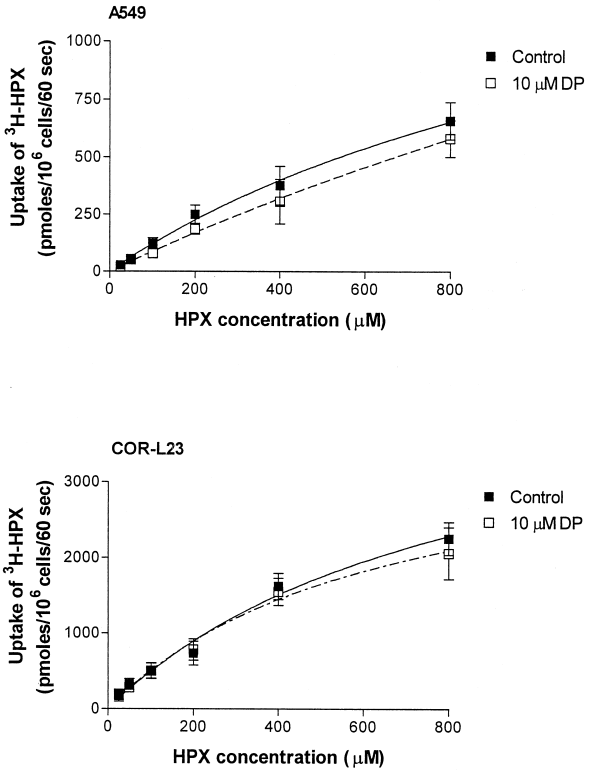

Fig. 2. The effect of DP on cellular uptake of HPX into A549 and COR-L23 cells. A549 and COR-L23 cells were incubated in DMSO (control, solid line), ■; or 10 μM DP (dotted line) □, for 5 min before HPX uptake was initiated by the addition of [^{3}H]HPX. Uptake was stopped after 60 sec by centrifugation through oil and cells were lysed with 3 M KOH prior to scintillation counting. Curves were fitted as described in Methods and points and curves are the mean ± SD of three determinations.

[10,16]. When the efficiency of HPX uptake in the A549 and COR-L23 cell lines was compared (T_{max}/K_t), COR-L23 cells were 5.5-fold more efficient at transporting HPX compared to A549 cells.

A K_i value for inhibition of HPX transport by DP in A549 cells of 6.8 μM was determined after 60 sec. K_i values of 10 and 0.16 μM for the inhibition of HPX transport by DP in Chinese hamster lung fibroblasts and human vascular endothelial cells (ECV 304) have been reported [11,27], and hence published DP K_i values provide a range which is not inconsistent with the result for A549 cells presented here. No inhibition of HPX uptake by DP was seen in COR-L23 cells. Similarly, others have reported that DP did not inhibit Na^+-independent HPX influx after 60 sec in LLC-PK$_1$ cells, although Na^+-dependent HPX uptake was inhibited with a K_i of 4.6 μM [16].

3.5. Sodium dependency of HPX uptake

To determine if HPX uptake was mediated via CNTs, as has been demonstrated in LLC-PK$_1$ cells [16], HPX uptake was measured in the presence or absence of sodium over a range of HPX concentrations in *ds* A549 and *di* COR-L23

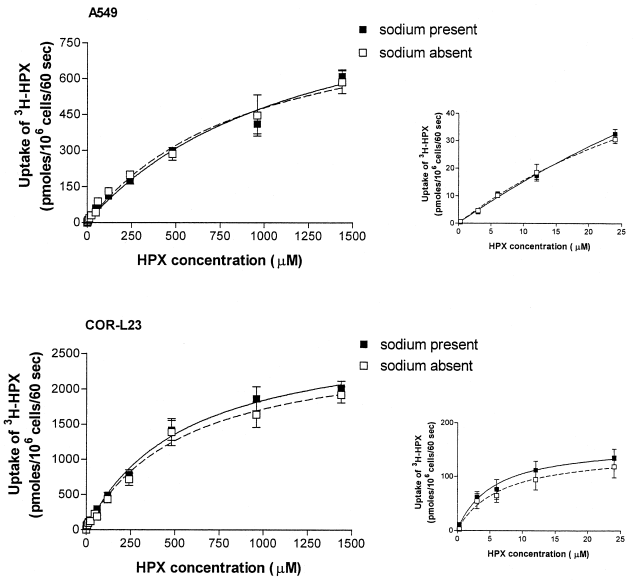

Fig. 3. The effect of sodium on HPX uptake in A549 and COR-L23 cell lines. A549 and COR-L23 cells were incubated with a range of [^{3}H]HPX concentrations in the presence of 140 mM NaCl (■) or 140 mM KCl (□) for 60 sec. Cells were lysed with 3 M KOH and [^{3}H]HPX uptake was determined. The inset graphs represent HPX uptake over low HPX concentration ranges. Curves were fitted as described in Methods and points and curves are the mean ± SD of three determinations.

cells after 60 sec. Even at low HPX concentrations, where sodium-dependent HPX uptake has been reported [16], the absence of sodium did not significantly reduce HPX transport (Fig. 3). Similarly, the absence of sodium did not significantly alter HPX K_t or T_{max} values in the two cell lines studied ($P > 0.5$, data not shown), suggesting that Na^+-dependent HPX transporters are not present in these cell lines.

3.6. Competition studies

To determine whether or not HPX enters cells via ENT2, HPX uptake was studied in the presence of TdR and 50 nM NBTI. As a positive control, the effect of the purine nucleoside inosine on TdR uptake was determined, since inosine is also a substrate for ENT2 [28]. In all four cell lines, the presence of 200 μM inosine reduced the uptake of 200 μM TdR. However, the effect of inosine was more marked in the two breast carcinoma cell lines, reducing ENT2-mediated control TdR uptake by 61 ± 18% ($P = 0.006$) in MCF7 cells and by 73 ± 17% ($P = 0.002$) in T-47D cells. In the lung cell lines, inosine inhibited TdR uptake in A549 cells by 23 ± 5% and by 34 ± 13% in COR-L23 cells. The presence of 200 μM HPX did not significantly reduce TdR uptake into A549 or COR-L23 cells; however, HPX did significantly reduce TdR uptake in the MCF7 and T-47D breast cell lines by 48 ± 13% ($P = 0.006$) and 41 ± 19% ($p = 0.01$), respectively.

The converse experiment, namely the effect of TdR and inosine on the uptake of HPX at 60 sec in the presence of 50 nM NBTI, was also conducted in all four cell lines. In the presence of NBTI, TdR and inosine appeared to reduce HPX uptake by about 20% in MCF7 and T-47D breast carcinoma cell lines, and by 15% in A549 lung carcinoma cells; however, the effect was not statistically significant in any of the cell lines.

3.7. HPLC analysis of intracellular HPX-derived material

To determine the extent of metabolism of HPX under the conditions of the uptake assay, intracellular HPX and metabolites were determined in cells incubated with 200 μM HPX for 60 sec. In all cell lines at 60 sec, the majority of the extractable radioactivity co-eluted with standard HPX. In the *di* COR-L23 and T-47D cell lines, the HPX peak comprised 96 ± 2% and 94 ± 1% of the cellular radioactivity, respectively, suggesting that metabolism of HPX was limited. In the *ds* A549 and MCF7 cell lines, somewhat greater conversion to other chemical species was observed, with 75 ± 3% and 79 ± 3% of the total radioactivity co-eluting with the HPX peak. The remaining radioactivity in the A549 and MCF7 cells had a mean retention time of 4 min, similar to retention time of AMP and IMP standards. The extent of HPX metabolism detected in these cells is similar to that observed in LLC-PK_1 cells, where 86% of the radioactivity was present as unmetabolised HPX following incubation of 100 μM [^{3}H]HPX for 1 min, as determined by thin-layer chromatography [16].

To investigate if HPX was being converted to purine nucleotides and then rapidly incorporated into RNA and DNA, radioactivity associated with cellular macromolecules in the perchloric acid pellet was determined. In all cell lines, the radioactivity of the pellet was <1.2% of the radioactivity contained in the supernatant. Thus, very little [^{3}H]HPX was incorporated into macromolecules during the limited time–course of the uptake experiments, and the majority of the radioactivity was present as unmetabolised HPX.

4. Discussion

The aim of the work presented here was to investigate potential mechanism(s) responsible for the differential sensitivity of HPX rescue from antipurine antifolate-induced growth inhibition to DP in human tumour cell lines. Non-carrier mediated diffusion may be excluded due to the different rates of HPX transport, the saturation kinetics, and the competition and inhibition effects observed with the four cell lines. Similarly, these studies have ruled out NBTI-sensitive HPX uptake (Fig. 1). Sodium-dependent HPX uptake was not observed in the two cell lines selected to represent the *ds* and *di* phenotypes, i.e. A549 and COR-L23, respectively (Fig. 3).

Earlier studies indicated that cells with DP-sensitive

HPX uptake also possessed a high proportion of ENT2, suggesting that HPX uptake was mediated via ENT2 in these cells [10]. Other cell types with less ENT2 were found to possess DP-insensitive nucleobase transporters [10]. In contrast, the results presented here indicate that there is no relationship between the percentage of ENT2 and the sensitivity of HPX rescue to DP; instead, cells with DP-insensitive HPX rescue were found to have higher rates of ENT2-mediated TdR uptake (Table 1).

Further studies of ENT2-mediated TdR and HPX uptake to determine whether HPX was a substrate for the ENT2 showed that HPX did not significantly inhibit ENT2-mediated TdR transport in A549 or COR-L23 cells. However, HPX inhibited TdR uptake by more than 40% in MCF7 and T-47D breast cell lines. When the converse experiments were performed, TdR and inosine inhibited HPX uptake in the breast cancer cells, albeit to a lesser extent than HPX inhibited TdR uptake. Together, these competition experiments provide evidence for a shared nucleobase/nucleoside transporter in MCF7 and T-47D cells.

A relationship between the temporal kinetics of HPX uptake and the sensitivity of HPX rescue to DP was observed in the four cell lines. HPX uptake was more rapid (reaching steady-state levels within 60 sec) in the two cell lines with *di* HPX rescue compared to the *ds* MCF7 and A549 cell lines, where a relatively slow linear increase in HPX levels was observed with time (Fig. 1). The high rate of HPX uptake in the *di* cell lines appears to correlate with the high rate of ENT2-mediated TdR uptake (Table 1).

From growth inhibition studies with antipurine antifolates [22,23], it was assumed that 10 μM DP would inhibit HPX uptake in the cell types with *ds* HPX rescue, but produce no inhibition of HPX uptake in cells with *di* HPX rescue. In accordance with these predictions, DP inhibited uptake of HPX into A549 and MCF7 cells by $>$ 48%. Although complete inhibition of HPX uptake was not achieved, it should be noted that the concentration of HPX in the transport assays was 200 μM, compared to 30 μM in growth inhibition studies. Presumably, the residual uptake was not sufficient to compensate for inhibition of *de novo* purine synthesis and maintain cell viability. Given the DP sensitivity of HPX uptake in the MCF7 cells and the competition between HPX and TdR uptake, it appears likely that HPX uptake occurs via ENT2 in these cells. The role of ENT2 in HPX uptake in A549 cells is not so clear, since although DP inhibited HPX uptake by 67% there was no clear evidence of overlap of HPX-uptake and ENT2-mediated TdR uptake by competition studies. It is also unlikely that HPX uptake is mediated via a DP-sensitive sodium dependent transporter as transport kinetics were the same in the presence and absence of sodium.

In T-47D cells, DP inhibited HPX uptake by 24% (Table 2), and there was an overlap with ENT2-mediated TdR uptake. Indeed, T-47D had the highest rate of ENT2-mediated TdR uptake and HPX inhibited TdR uptake by 41%. This suggests that HPX uptake by T-47D cells may also be via DP-sensitive ENT2. One possible mechanism is that T-47D cells possess a subtype of ENT2 that, like those found in Novikoff hepatoma and ECV 304 cells, has a higher affinity for HPX [1,2]. Consistent with this proposal. previous studies have suggested a multiplicity in ENT2 subtypes [28]. Despite HPX transport being inhibited by DP in T-47D cells, the higher HPX uptake in these cells, compared to MCF7 cells, may provide sufficient HPX for rescue from antipurine antifolate-induced growth inhibition.

In contrast, HPX uptake into COR-L23 cells was highly efficient and rapid, and the lack of DP inhibition ensured that these cells were rescued by HPX from the effect of antipurine antifolates in the presence of DP. Absence of DP inhibition and no clear evidence of competition between HPX-uptake and ENT2-mediated TdR uptake indicates that HPX uptake occurs via a separate DP-insensitive HPX carrier in these cells.

In summary, these studies have demonstrated differences in the mode of HPX transport in four human tumour cell lines displaying differential sensitivity to the combination of antipurine antifolates, HPX, and DP. Specifically, the two *di* cell lines accumulated HPX more rapidly and to a greater extent than the *ds* cells. An overlap of HPX-and ENT2-mediated TdR transport was observed in the two breast cancer cell lines, but not the lung cancer cells. Inhibition of HPX uptake by DP and overlap with ENT2-mediated TdR transport suggest that ENT2 is the predominant HPX carrier in MCF7 cells. Conversely, lack of inhibition by DP and no overlap with nucleoside transport indicate a separate DP-insensitive HPX transporter in COR-L23 cells. HPX uptake in A549 cells appears to be via a DP-sensitive transporter that does not coincide with ENT2 or a sodium-dependent transporter, while in T-47D cells HPX uptake is only modestly inhibited by DP despite evidence of overlap with ENT2. It appears that human tumour cells may express one or more of a variety of HPX transporters and that the ability of DP to prevent HPX rescue in antipurine antifolate-treated cells is dependent on the transporter type expressed. Transporter phenotype is likely to have profound implications for combination chemotherapy with antifolates and nucleoside transport inhibitors.

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References

[1] Plagemann PG, Wohlhueter RM. Hypoxanthine transport in mammalian cells: cell type-specific differences in sensitivity to inhibition by dipyridamole and uridine. J Membr Biol 1984;81:255–62.
[2] Belt JA. Heterogeneity of nucleoside transport in mammalian cells. Mol Pharmacol 1983;24:479–84.

[3] Crawford CR, Patel DH, Naeve C, Belt JA. Cloning of the human equilibrative, nitrobenzylmercaptopurine riboside (NBMPR)-insensitive nucleoside transporter *ei* by functional expression in a transport-deficient cell line. J Biol Chem 1998;273:5288–93.
[4] Griffiths M, Beaumont N, Yao SY, Sundaram M, Boumah CE, Davies A, Kwong FY, Coe I, Cass CE, Young JD, Baldwin SA. Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. Nat Med 1997;3:89–93.
[5] Griffiths M, Yao SY, Abidi F, Phillips SE, Cass CE, Young JD, Baldwin SA. Molecular cloning and characterisation of a nitrobenzylthioinosine-insensitive (*ei*) equilibrative nucleoside transporter from human placenta. Biochem J 1997;328:739–43.
[6] Baldwin SA, Mackey JR, Cass CE, Young JD. Nucleoside transporters: molecular biology and implications for therapeutic development. Mol Med Today 1999;5:216–24.
[7] Ritzel MW, Yao SY, Huang MY, Elliott JF, Cass CE, Young JD. Molecular cloning of functional expression of cDNAs encoding a human Na^+-nucleoside cotransporter (hCNT1). Am J Physiol 1997; 41:C707–14.
[8] Wang J, Su SF, Dresser MJ, Schaner ME, Washington CB, Giacomini KM. Na^+-dependent purine nucleoside transporter from human kidney: cloning and functional characterisation. Am J Physiol 1997;42: F1058–65.
[9] Ritzel MW, Yao SY, Ng AM, Mackey JR, Cass CE, Young JD. Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na^+/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine. Mol Membr Biol 1998;15:203–11.
[10] Plagemann PG, Wohlhueter RM, Woffendin C. Nucleoside and nucleobase transport in animal cells. Biochim Biophys Acta 1988; 947:405–43.
[11] Slaughter RS, Barnes EM. Hypoxanthine transport by Chinese hamster lung fibroblasts: kinetics and inhibition by nucleosides. Arch Biochem Biophys 1979;197:349–55.
[12] Aronow B, Allen K, Patrick J, Ullman B. Altered nucleoside transport in mammalian cells selected for resistance to the physiological effects of inhibitors of nucleoside transport. J Biol Chem 1985;260:6226–33.
[13] Domin BA, Mahony WB, Zimmerman TP. Purine nucleobase transport in human erythrocytes. Reinvestigation with a novel "inhibitor-stop" assay. J Biol Chem 1988;263:9276–84.
[14] Domin BA, Mahony WB, Zimmerman TP. Transport of 5-fluorouracil and uracil into human erythrocytes. Biochem Pharmacol 1993; 46:503–10.
[15] Kraupp M, Marz R, Prager G, Kommer W, Razavi M, Baghestanian M, Chiba P. Adenine and hypoxanthine transport in human erythrocytes: distinct substrate effects on carrier mobility. Biochim Biophys Acta 1991;1070:157–62.
[16] Griffith D, Jarvis SM. High affinity sodium-dependent nucleobase transport in cultured renal epithelial cells (LLC-PK_1). J Biol Chem 1993;268:20085–90.
[17] Akerman R, Jarvis SM. Nucleobase transport in cultured renal epithelial cells. Biochem Soc 1995;23:29S.
[18] Bronk JR, Hastewell JG. The transport of pyrimidines into tissue rings cut from rat small intestine. J Physiol 1987;382:475–88.
[19] Beardsley GP, Moroson BA, Taylor EC, Moran RG. A new folate antimetabolite, 5,10-dideaza-5,6,7,8-tetrahydrofolate is a potent inhibitor of *de novo* purine synthesis. J Biol Chem 1989;264:328–33.
[20] Mendelsohn LG, Shih C, Schultz RM, Worzalla JF. Biochemistry and pharmacology of glycinamide ribonucleotide formyltransferase inhibitors: LY309887 and lometrexol. Invest New Drugs 1996;14:287–94.
[21] Boritzki TJ, Barlett CA, Zhang C, Rowland EF, Margosiak SA, Palmer CL, Romines WH, Jackson RC. AG2034: a novel inhibitor of glycinamide ribonucleotide formyltransferase. Invest New Drugs 1996;14:295–303.
[22] Turner RM, Aheme GW, Curtin NJ. Selective potentiation of lometrexol growth inihibition by dipyridamole through cell-specific inhibition of hypoxanthine salvage. Cancer Res 1997;51:2346–52.
[23] Marshman E, Newell DR, Calvert AH, Dickinson AM, Patel HR, Campbell FC, Curtin NJ. Dipyridamole potentiates antipurine antifolate activity in the presence of hypoxanthine in tumour cells but not in normal tissues *in vitro*. Cancer Res 1988;11:2895–902.
[24] Chen TR. *In situ* detection of mycoplasma contamination in cell culture by fluorescent Hoechst 33258 stain. Exp Cell Res 1977;104: 255–62.
[25] Wohlhueter RM, Marz R, Graff JC, Plagemann PG. A rapid-mixing technique to measure transport in suspended animal cells: applications to nucleoside transport in Novikoff rat heptoma cells. Methods Cell Biol 1978;20:211–36.
[26] Paterson AR, Kolassa N, Cass CE. Transport of nucleoside drugs in animal cells. Pharmacol Ther 1981;12:515–36.
[27] Osses N, Pearson JD, Yudilevich DL, Jarvis SM. Hypoxanthine enters human vascular endothelial cells (ECV 304) via the nitrobenzylmioinosine-insensitive equilibrative nucleoside transporter. Biochem J 1996;317:843–48.
[28] Griffith DA, Jarvis SM. Nucleoside and nucleobase transport systems of mammalian cells. Biochim Biophys Acta 1996;1286:153–81.