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Compensatory effects of the human nucleoside transporters on the response to nucleoside-derived drugs in breast cancer MCF7 cells

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ABSTRACT

Nucleoside transporters (NTs) are involved in the cytotoxicity and transcriptomic response induced by nucleoside analogues. A relationship between the expression of nucleoside transporters and response to therapy has been demonstrated in solid tumours, although the pattern of such expression is highly variable. Thus, a question is whether the transporter expression pattern rather than specific NT proteins might better explain the ability of tumour cells to respond to nucleoside-derived drug therapy. In this study we used the breast cancer cell lines MCF7 and MCF7–hCNT1 (stably transfected with hCNT1) to determine whether hCNT1 expression can complement hENT1 functional loss in the cytotoxicity and transcriptomic response triggered by nucleoside analogues. Expression of hCNT1 slightly increased cell sensitivity to 5′-deoxy-5-fluorouridine (5′-DFUR). Inhibition of the endogenous equilibrative activity blocked 5′-DFUR cytotoxicity in MCF7 cells, but not in MCF7–hCNT1 cells. Moreover, under equilibrative transport inhibition conditions, induction of some transcriptional targets of 5′-DFUR was blocked in MCF7 cells, whereas ENT-inhibition had no effect on the transcriptional response to 5′-DFUR in MCF7–hCNT1 cells. To confirm the role of hCNT1 in 5′-DFUR treatment, a panel of nucleoside derivatives suitable for hCNT1-inhibition was obtained. The molecule T-Ala inhibited hCNT1-mediated transport. Furthermore, the cytotoxic action of 5′-DFUR and the transcriptional changes produced by this nucleoside analogue were partially inhibited by T-Ala in MCF7–hCNT1 cells. These results show a link between NT function and the pharmacogenomic response to nucleoside analogues and further support the hypothesis that the expression pattern rather than specific transporters determines the cytotoxic effect of nucleoside derivatives.

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1. Introduction

Nucleoside-derived drugs are currently used in the treatment of a variety of solid tumours and lymphoproliferative diseases. These molecules must be transported into cells to exert their cytotoxic action and, for that purpose, they are targeted to plasma membrane proteins involved in the salvage pathway of natural nucleosides and other physiological processes. Two gene families encode these transporter proteins, SLC28 and SLC29, the former encoding human Concentrative Nucleoside Transporters (hCNTs), the latter human Equilibrative Nucleoside Transporters (hENTs) [1,2]. The hENT members 1 and 2 show broad selectivity for natural nucleosides and, to some extent, for nucleoside-derived drugs, and specificity is relatively low particularly when compared with the hCNT protein family. hCNT transporters are characterized by a more selective pattern of substrate recognition (particularly evident for hCNT1 and hCNT2) and lower apparent K_m values for their natural substrates and nucleoside-derived drugs with antitumour activity.

The probable relationship between nucleoside transporter expression and response to therapy has been recently examined in our laboratory and others, both in solid tumours and in lymphoproliferative malignancies. It has been shown that hENT1 protein correlates with *ex vivo* sensitivity to gemcitabine in Mantle Cell Lymphoma (MCL) cells [3], whereas in a lymphoproliferative disease, such as Chronic Lymphocitic Leukemia (CLL), not characterized by deregulated proliferation but rather by impaired apoptosis, *ex vivo* sensitivity to fludarabine correlates with hENT2 transporter expression and related functional activity [4,5]. In breast cancer, long-term disease-free survival after surgery in patients who underwent CMF (cyclophosphamide, methotrexate, 5-fluorouracil (5-FU)) therapy, inversely correlates with hCNT1 expression [6], possibly as a result of promoting nucleoside salvage in a context in which 5-FU impairs intracellular nucleotide metabolism. Moreover, hENT1 expression correlates with survival in pancreatic adenocarcinoma patients under gemcitabine treatment as a single therapy [7,8].

Nucleoside-derived drug cytotoxicity is associated with selective changes in gene expression. This transcriptomic response involves a variety of p53-dependent genes and some key elements of the cell machinery implicated in cell cycle progression and apoptosis [9,10]. Cytotoxicity assays are routinely performed to analyze the anticancer potential of nucleoside-derived drugs and other types of molecules. They involve exposing cells to drugs for relatively long periods of time, under conditions that might not reflect the *in vivo* situation of the patient. Nevertheless, when analysing the mechanism by which nucleoside-derived drugs exert their action in MCF7 cells, we were recently able to demonstrate that relatively short periods of incubation (90 min) with the 5-FU precursor and capecitabine metabolite, 5′-DFUR, elicit transcriptomic responses similar to those obtained after longer exposures (i.e., 24 h). In a cell system (MCF7) in which only hENT1 and hENT2 appear to be functional, most of the transcriptomic response is blocked by pharmacological inhibition of hENT1 [11], which indicates a relevant role for this particular transporter isoform in the nucleoside-triggered transcriptomic response. However, tumour biology is certainly more intricated and, in fact, evidence has been provided of complex nucleoside transporter patterns in gynaecological tumours, in which hENT1, hENT2 and hCNT1 expression has been analyzed using a high throughput approach by means of tissue arrays [12]. The question thus arises as to whether the response of tumour cells to nucleoside-derived drug therapy is better explained in terms of overall transporter expression pattern, or by the individual NT proteins.

In this study we have addressed this issue by engineering MCF7 cells to express hCNT1. We thereby demonstrated that heterologous expression of this transporter protein in itself promotes chemosensitivity to 5′-DFUR, and even contributes to maintaining the transcriptomic response to this drug under conditions in which hENT-related activity has been blocked.

2. Materials and methods

2.1. Reagents

Uridine ([5,6-^{3}H], 35–50 Ci/mmol) was purchased from Amersham Biosciences (UK). cytidine ([5-^{3}H(N)], 21.5 Ci/mmol) and guanosine ([8-^{3}H(N)], 7 Ci/mmol) were from Moravek Biochemicals (CA, USA). Uridine, cytidine, guanosine and dipyridamole were obtained from Sigma–Aldrich (Saint Louis, MO, USA).

2.2. Synthesis of T-Ala and T-NH-Ala

2.2.1. 3′-O-L-Alanyl-thymidine (T-Ala)

To a solution of 5′-O-DMT-thymidine (1.0 g, 1.84 mmol, Syngen) in HCl-free dichloromethane was added a solution of Boc-L-Ala-OH (418 mg, 2.21 mmol, Novabiochem), *N*,*N*′-dicyclohexylcarbodiimide (456 mg, 2.21 mmol) and *N*,*N*-dimethylaminopyridine (56 mg, 0.46 mmol) in HCl-free dichloromethane. After 90 min stirring at room temperature, the mixture was chilled, and *N*,*N*′-dicyclohexylurea was filtered and washed. Aqueous treatment of the filtrates (10% aq tartaric acid, 10% aq Na_2CO_3 and brine) afforded pure 5′-O-DMT-T-3′-O-L-Ala-Boc. The DMT and Boc groups were removed by treatment with 4 ml of a 40% solution of trifluoroacetic acid (TFA) in dichloromethane at room temperature for 15 min. T-Ala was obtained after precipitation from *tert*-butyl methyl ether, and purification by medium pressure liquid chromatography (Vydac C18, solvent A: 0.05% TFA in water, solvent B: 0.05% TFA in water/acetonitrile 65:35, 500 ml of each solvent). 50% overall yield; anal HPLC (Nucleosil C18, 250 mm × 5 mm, 10 μm, linear gradient from 5 to 45% of B in 30 min, A: 0.045% TFA in water, B: 0.036% TFA in acetonitrile); t_R: 10.4 min (purity 97%); ^{1}H NMR (D_2O, 200 MHz) δ: 7.43 (1H, d, *J* 1 Hz, H6), 6.08 (1H, m, H1′), 5.21 (1H, m, H3′), 4.00 (2H, m, H4′, Hα), 3.59 (2H, m, H5′), 2.25 (2H, m, H2′), 1.64 (3H, d, *J* 1.2 Hz, CH3 T), 1.35 (3H, d, *J* 7.2 Hz, Hβ); ^{13}C NMR (D_2O, 200 MHz) δ: 169.2, 165.7, 151.0, 136.5, 111.0, 84.4, 83.6, 76.0, 60.5, 48.1, 35.3, 14.4, 11.0; FAB-MS (magic bullet, positive mode): *m*/*z* 627.3 $[2M + H]^+$, 352.4 $[M + K]^+$, 336.4 $[M + Na]^+$, 314.4 $[M + H]^+$, calc. M (neutral molecule) 313.3.

2.2.2. 3′-Amino-3′-deoxythymidine (T-NH$_2$)

3′-Azido-3′-deoxythymidine (AZT, 500 mg, 1.87 mmol, Sigma) was dissolved in pyridine and treated with triphenylphosphine (736.2 mg, 2.80 mmol) in N_2 atmosphere for 40 min

(gas evolution was observed). 15 ml of a 32% aqueous ammonia solution was added, and the mixture was allowed to react overnight. Solvent removal was followed by addition of water and extraction with dichloromethane. Concentration of the aqueous solution and co-evaporation with acetonitrile yielded T-NH_2 as a white solid. TLC analysis showed that the aqueous phase did not contain other organic material. 80% overall yield; ^{1}H NMR (DMSO-d_6, 200 MHz) δ: 7.75 (1H, s, H6), 6.08 (1H, m, H1′), 4.96 (1H, broad peak, HO 5′), 3.35–3.75 (4H, m, H3′, H4′, 2H5′), 2.05 (2H, m, H2′), 1.76 (3H, s, CH_3 T); CI-MS (positive mode): *m*/z 241.9 $[M + H]^+$, calc. M (neutral molecule) 241.2.

2.2.3. *3′-N-(L-Alanyl)-3′-amino-3′-deoxythymidine (T-NH-Ala)*

A mixture of T-NH_2 (200 mg, 0.83 mmol), Boc-L-Ala-OSu (285 mg, 0.995 mmol, Novabiochem) and *N*-ethyldiisopropylamine (169.2 μl) was dissolved in tetrahydrofuran (10 ml) and methanol (4 ml), and stirred overnight at room temperature. After concentration *in vacuo*, ethyl acetate was added, and aqueous work-up (10% tartaric acid and brine) afforded T-NH-Ala-Boc. Further analysis showed that T-NH-Ala-Boc was present in both the organic and aqueous phases, which explains the low synthesis yield (see below). The Boc group was removed by reaction with TFA (30% in dichloromethane) for 30 min at room temperature. T-NH-Ala was obtained after precipitation from *tert*-butyl methyl ether and purification by medium pressure liquid chromatography (Vydac C18, solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in water/acetonitrile 20:80, 500 ml of each solvent). 6% overall yield; anal HPLC (Kromasil C18, 100 mm × 4 mm, 5 μm, linear gradient from 0 to 80% of B in 10 min, A: 0.045% TFA in water, B: 0.036% TFA in acetonitrile): t_R: 8.9 min (purity 99%); ^{1}H NMR (D_2O, 200 MHz) δ: 7.53 (1H, s, H6), 6.05 (1H, m, H1′), 4.40–3.50 (5H, m, H3′, H4′, Hα, H5′), 2.28 (2H, m, H2′), 1.72 (3H, s, CH_3 T), 1.36 (3H, d, J 7.2 Hz, Hβ); ^{13}C NMR (D_2O, 200 MHz) δ: 170.0, 166.0, 151.0, 136.9, 110.8, 84.2, 83.3, 60.1, 48.5, 48.3, 35.7, 16.0, 11.0; CI-MS (positive mode): *m*/z 313.0 $[M + H]^+$, calc. M (neutral molecule) 312.3.

2.3. Cell culture and nucleoside uptake measurements

The human breast carcinoma cell lines MCF7 (HTB-22, ATCC-LGC Promochem Partnership, USA), and hCNT1–MCF7 which expresses the human concentrative pyrimidine nucleoside transporter, hCNT1, were used. The MCF7–hCNT1 clone was generated by stable transfection of MCF7 cells with a pcDNA3 vector incorporating hCNT1 cDNA, cloned from human foetal liver (GenBank accession number U62966) [13]. Putative hCNT1-expressing clones were selected using geneticin treatment, and several independent clones were checked. MCF7–hCNT1 cells showed a typical hCNT1 activity pattern, characterized by high-affinity uptake of pyrimidine nucleosides and a lack of transport of purine nucleosides. Cells were routinely cultured in DMEM supplemented with 10% foetal bovine serum (GIBCO-BRL, Grand Island, NY), 2 mM glutamine and a mixture of antibiotics (100 U penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml fungizone). Cells were maintained as monolayer cultures at 37 °C in an atmosphere of 5% CO_2 and subcultured every 4–5 days.

hCNT1 activity was measured by incubating cell monolayers in an uptake buffer (5.4 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 10 mM HEPES), supplemented with either sodium or choline chloride (137 mM), in which the labelled pyrimidine nucleoside was added at a final concentration of 1 μM (specific activity 1 μCi/nmol). This method allows the calculation of Na^+-dependent uptake rates that are exclusively accounted for by the hCNT1 transporter. Incubations were stopped by rapid aspiration of the uptake buffer followed by immediate washing with a cold stop solution, as previously described [14].

2.4. Expression of CNT1 in Xenopus laevis oocytes

The hCNT1 cDNA was subcloned into a Bluescript vector, which was then linearized with *Xba*I and cRNA synthesized using the Ambion T3MEGAscript kit (Austin, TX, USA) in the presence of m7G(5′)ppp(5′)G. Fifty nanograms of cRNA was injected into *Xenopus laevis* oocytes, which were then maintained at 18 °C in Barth's medium for 3–5 days. The transportability/interaction of the potential hCNT1-blockers synthesized was then assessed, taking advantage of the fact that hCNT1 function is associated with substrate-induced Na^+ inward currents, using the two electrode voltage clamp technique previously described [15,16].

2.5. MTT cytotoxicity assays

The cytotoxicity assays were performed by seeding MCF7 and MCF7–hCNT1 cells at a density of 20,000 cells/cm^2 in 96-well culture plates. Twenty-four hours after seeding, cultures were exposed for 90 min to increasing concentrations of 5′-DFUR or 5-FU (from 10 μM to 3 mM), either in the presence or in the absence of 10 μM dipyridamole, 100 μM T-Ala and 100 μM T-NH-Ala. Dipyridamole was pre-incubated for 15 min before drug addition. Viability was assessed 48 h after the addition of the drugs using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At 48 h, 100 μl MTT reagent (7.5 mg/ml) was added to each well after removal of medium, and incubated for 45 min at 37 °C. Subsequently, the MTT reagent was discarded and the dark blue formazan crystals were solubilized in DMSO. The absorbance of formazan was measured photometrically at 595 nm using a microplate reader. The number of surviving cells is directly proportional to the quantity of the formazan product created. Data were fitted to a dose–response curve, using GraphPad Prism 4.0 software (GraphPad Sofware, Inc., San Diego, CA) to obtain the IC_{50}.

2.6. RNA isolation and quantitative RT-PCR

MCF7 cells were seeded at 20,000 cells/cm^2 in 100 mm dishes. After 24 h, cells were treated for 90 min with 5′-DFUR (250 μM) either in the presence or absence of 10 μM dipyridamole, 100 μM T-Ala and 100 μM T-NH-Ala. Twenty-four hours after the commencement of treatment, cells were trypsinized and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of total DNase-treated RNA was used to generate cDNA, using M-MLV Reverse Transcriptase (Promega Biotech) and random hexamers (Amersham-Pharmacia) for reverse transcription.

Quantitative real-time PCR was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), following the instructions of the manufacturer. Assays-on-Demand Taqman probes (Applied Biosystems) of CDKN1A/p21, TNFRSF6/FAS, AQP3, RPL3, RRM2B and GAPDH were used. Relative quantification of gene expression was performed as described in the *Taq*Man user's manual, with GAPDH as an internal control.

2.7. Immunoblotting

Protein extracts were obtained for the analysis of hCNT1 expression. Briefly, cells were lysed in 1 ml of phosphate buffer (sodium phosphate 0.5 mM, EDTA 0.1 mM, pH 7) containing a protease inhibitor cocktail (Complete MINI, Roche). After sonication for 15 s, cells were centrifuged at 16,000 × *g* for 45 min at 4 °C. The remaining pellets were lysed in 100–150 μl of the same buffer. Monospecific polyclonal antibody against hCNT1 was raised and characterized in our laboratory [13].

For Bax analysis, control and treated cells were lysed in RIPA buffer containing a protease inhibitor cocktail. Bax expression was determined using a polyclonal anti-Bax antibody (Santa Cruz Biotechnology, CA).

Five to forty micrograms of each protein extract was separated on 10–12% polyacrylamide gels and transferred to Immobilon-P (Millipore, Bedford, MA) membranes. Following antibody incubation, proteins were detected using secondary antibodies conjugated to horseradish peroxidase and an enhanced chemiluminescence (ECL) detection kit (Amersham, Buckinghamshire, UK). Protein loading was confirmed with α-tubulin (Oncogene Research, Boston, MA). Densitometric analysis was performed using Phoretix 1D Software. Semi-quantitative analysis was carried out by calculating the densitometry ratios relative to α-tubulin.

2.8. Statistical analysis

The unpaired Student's *t*-test was used to compare experimental data. This analysis has been carried out using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Heterologous expression of hCNT1 in MCF7 cells

Since nucleoside uptake in MCF7 cells was found to be mediated only by equilibrative transporters (data not shown), we heterologously expressed hCNT1 in this cell line. Transfected cells were selected for G418 resistance and screened for Na^+-dependent nucleoside transport activity, and the clone with the highest concentrative uridine uptake rates (MCF7–hCNT1) was selected for further experiments. For comparative purposes, MCF7 cells transfected with the pcDNA3 vector were also obtained. Uridine uptake in MCF7–hCNT1 cells was linear for up to 10 min, and a significant fraction of this transport activity appeared to be sodium-dependent (Fig. 1A). Uridine uptake in MCF7 and MCF7-pcDNA3 cells was exclusively mediated by equilibrative nucleoside transporters (data not shown). No changes in the endogenous equilibrative activity was observed as a result of hCNT1 heterologous expression in MCF7–hCNT1 cells (Fig. 1B), in agreement with the unaltered hENT-type mRNA levels (not shown). To confirm the pyrimidine-preferring nature of the activity evident in MCF7–hCNT1 cells, cytidine and guanosine were used as substrates. Data shown in Fig. 1B demonstrates the pyrimidine-preferring nature of the sodium-dependent component (the classical feature of the hCNT1 transporter). Furthermore, as deduced from the Western blot analysis shown in Fig. 1C, MCF7–hCNT1 cells overexpressed the hCNT1 protein, although both MCF7 and MCF7-pcDNA3 cells did also express hCNT1 protein at low

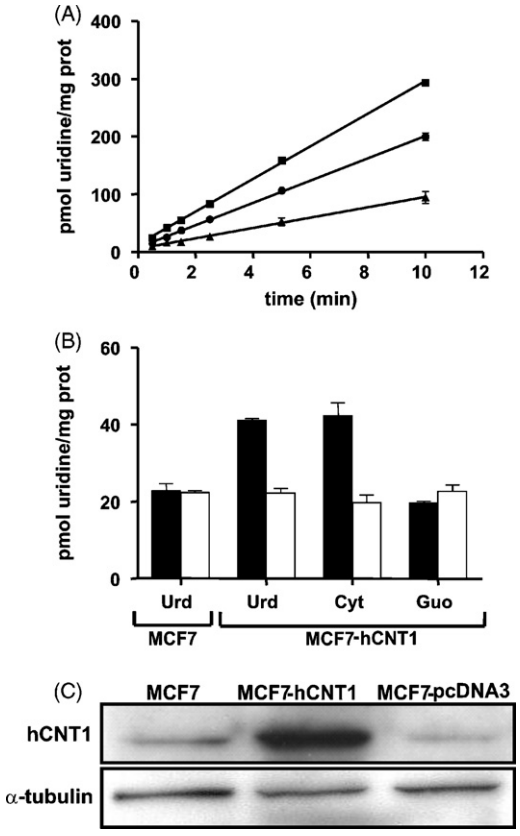

Fig. 1 – Characterization of MCF7 cell line stably transfected with the hCNT1 gene. (A) Time course of uridine uptake into MCF7 cell line stably transfected with hCNT1. Cells were incubated with 1 μM uridine either in NaCl (■) or choline chloride medium (●). Concentrative uptake (Na^+-dependent uptake) (▲) was calculated as the transport rate in the presence of Na^+ minus the rate in its absence (choline medium). (B) To confirm the pyrimidine-preferring nature of the transporter activity, cytidine and guanosine were used as substrates. MCF7 cell types (wild type and hCNT1) were incubated with 1 μM nucleoside either in NaCl (closed) or choline chloride (open) media for 60 s. Results are the mean ± S.E.M. of three independent experiments. (C) Western blot analysis of hCNT1. Forty micrograms of total lysate extracts was analyzed with CNT1 antibody. α-Tubulin is used as loading control. A representative Western blot of three experiments, each performed with independent samples, is shown.

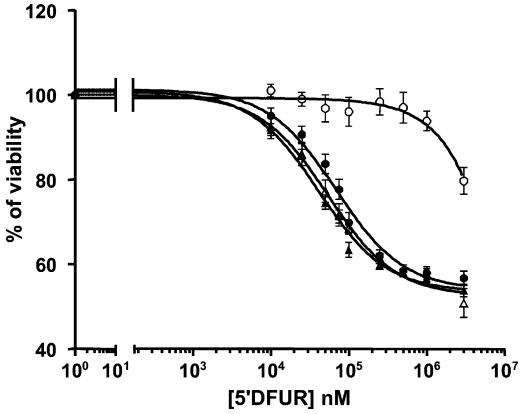

Fig. 2 – Cytotoxicity of 5′-DFUR in MCF7 and MCF7–hCNT1 cells. Cell viability was assessed by the MTT assay 48 h after a 90-min exposure to 5′-DFUR. Twenty-four hours after seeding MCF7 (●) or MCF7–hCNT1 (▲) cells were treated for 90 min with increasing concentrations of the drug in the absence (solid) or the presence (open) of the equilibrative nucleoside transporter inhibitor dipyridamole. Results are the mean ± S.E.M. of five to seven independent experiments.

levels. Although these two cell lines also expressed hCNT1-related mRNA (data not shown), we did not detect any sodium-dependent activity. For this reason, MCF7–hCNT1 cells can be considered a suitable model for analysis of the contribution of hCNT1 protein to nucleoside-derived cytotoxicity in a cell background with null hCNT1-related transport activity.

3.2. *Effect of hCNT1 expression on cell sensitivity to 5′-DFUR*

To assess the relationship between transport and cellular toxicity, the effect of heterologous hCNT1 expression on the sensitivity of MCF7 cells to 5′-DFUR was analyzed. 5′-DFUR cytotoxicity was measured 48 h after a 90-min treatment with increasing concentrations of the drug (Fig. 2). The expression of hCNT1 results in sensitization of cells to 5′-DFUR treatment by a factor of almost two (Table 1).

To determine the putative role of the hCNT1 transporter in 5′-DFUR sensitivity, the endogenous equilibrative nucleoside transporters were inhibited with 10 μM dipyridamole (Fig. 2). Inhibition of equilibrative transporters induced significant resistance to 5′-DFUR in wild-type MCF7 cells, but did not significantly alter the IC_{50} value for 5′-DFUR in MCF7–hCNT1 cells (Table 1). Inhibition of hENT1 using NBTI similarly produced a four-fold increase in the IC_{50} value for 5′-DFUR in MCF7 cells, but did not alter 5′-DFUR-induced cytotoxicity in MCF7–hCNT1 cells (data not shown). Similar results to those in MCF7 cells were obtained using MCF7-pcDNA3 cells (data not shown). Furthermore, the hCNT1-mediated sensitivity was specific for nucleoside derivatives, since expression of hCNT1 and equilibrative transport inhibition with dipyridamole did not affect sensitivity to the nucleobase 5-FU (not shown).

Table 1 – IC_{50} values for 5′-DFUR cytotoxicity

	Dipyridamole (−)	Dipyridamole (+)
MCF7	72.2 ± 7.8 μM	>3 mM
MCF7–hCNT1	42.1 ± 6.2 μM**	45.1 ± 6.2 μM

Statistical significance was assessed using the Student's *t* test: panel A: **$P < 0.01$.

3.3. *Synthesis and biological evaluation of hCNT1 inhibitors*

The choice of a single hCNT1-expressing clone to analyze nucleoside-derived cytotoxicity does not rule out the possibility that a change in drug sensitivity is the result of cell adaptations (i.e., metabolism) other than the gain of hCNT1 function. Thus, we sought to block hCNT1-related activity in order to provide more definitive evidence for its role in 5′-DFUR-associated cytotoxicity.

Unfortunately, there are no specific inhibitors of concentrative nucleoside transporters. Thus, a panel of potential hCNT1-blockers were synthesized, including some obtained by modifying the 3′ hydroxyl group of the sugar, which is known to be required for substrate recognition [17,18]. The structures of two such compounds are shown in Fig. 3A.

The interaction between hCNT1 and the synthesized compounds, T-Ala and T-NH-Ala, was assessed in MCF7–hCNT1 cells by monitoring the inhibition of 1 μM Na^+-dependent uridine uptake triggered by different concentrations of these analogues (Fig. 3B). Although T-Ala and T-NH-Ala differ only in the chemical bond between the nucleoside and the amino acid, T-Ala but not T-NH-Ala inhibited hCNT1 transporter related activity. The IC_{50} value of T-Ala inhibition of hCNT1 was 20.9 μM, close to that obtained for natural nucleosides. However, although T-Ala produced almost 80% inhibition of hCNT1-mediated transport at 100 μM, at this concentration it also exhibited a minimal (less than 15%) inhibition of the uptake mediated by endogenous equilibrative transporters (Fig. 3C), which means that only partial inhibition of hCNT1 activity could be achieved by using this novel inhibitor. To test whether T-Ala and T-NH-Ala were substrates of hCNT1, we analyzed the ability of these two compounds to generate substrate-driven Na^+-inward currents in *X. laevis* oocytes expressing the transporter, using the two-electrode voltage clamp technique. The results showed that neither T-Ala nor T-NH-Ala induced an inward current (not shown) indicating that they are not translocated, and consequently that they are not substrates of hCNT1. Therefore, we conclude that a novel compound has been synthesized, that is not transported by hCNT1 but it is recognized with relatively high affinity, thus blocking hCNT1-function.

Sensitivity of MCF7–hCNT1 cells to 5′-DFUR was analyzed using both synthesized compounds. To further dissect the effect of hCNT1 expression functionally, endogenous equilibrative transporters were inhibited using dipyridamole, as in Fig. 2. Thus, MCF7–hCNT1 cells were treated for 90 min with 10 μM dipyridamole and increasing concentrations of 5′-DFUR, either in the presence or in the absence of 100 μM T-Ala or T-NH-Ala. Cytotoxicity was measured at 48 h (Fig. 3D). Inhibition

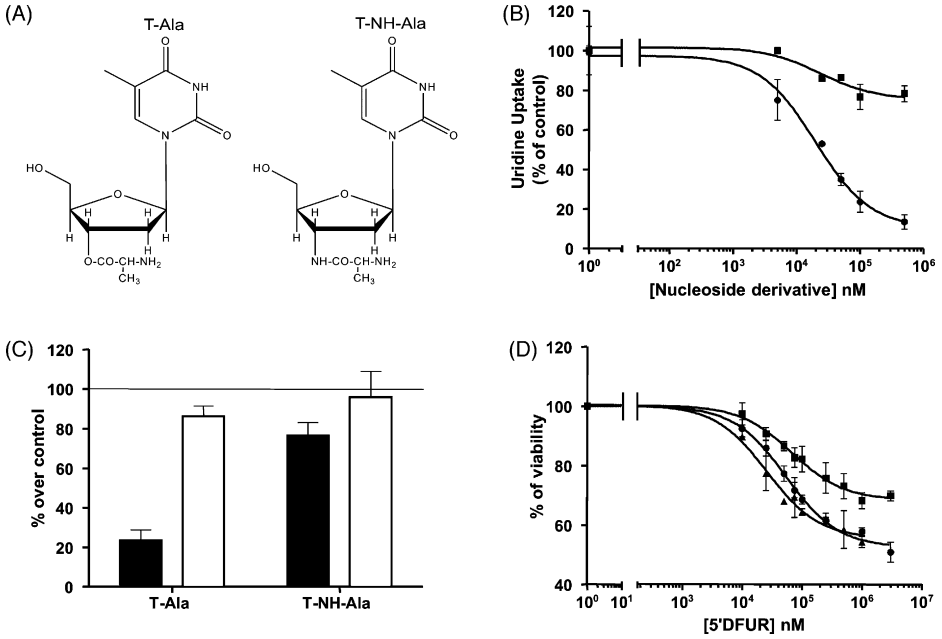

Fig. 3 – Structures and biological evaluation of hCNT1-blockers. (A) Structures of two putative blockers of hCNT1 activity. (B) Inhibition of hCNT1-mediated uridine uptake by increasing concentrations of T-Ala (●) and T-NH-Ala (■). The IC_{50} values were 20.9 ± 6.25 μM and >1 mM, respectively. (C) Inhibition of concentrative (black) and equilibrative (white) uridine uptake by 100 μM T-Ala and T-NH-Ala in MCF7–hCNT1 cells. Data are expressed as percentage of uptake versus control (absence of inhibitor) value. (D) Inhibition of 5′-DFUR cytotoxicity with T-Ala and T-NH-Ala in MCF7–hCNT1 cells. Cells were treated with 10 μM dipyridamole and increasing concentrations of 5′-DFUR (●) in the presence of 100 μM T-Ala (■) or T-NH-Ala (▲). Cell viability was assessed by the MTT assay 48 h after exposure.

of hCNT1 with T-Ala induced a significant resistance to 5′-DFUR, whereas T-NH-Ala did not significantly alter the IC_{50} value. Moreover, neither T-Ala nor T-NH-Ala affected 5′-DFUR sensitivity in MCF7 cells (not shown).

3.4. *Gene expression after 5′-DFUR treatment*

To further study the role of nucleoside transporters in the response to 5′-DFUR treatment, we selected a panel of upregulated genes, namely those encoding CDKN1A/p21, TNFRF6/FAS, RRM2B, AQP3 and a single downregulated gene, that of RPL3, previously identified as 5′-DFUR targets [11]. We then monitored changes in their corresponding mRNA levels in both MCF7 and MCF7–hCNT1 cells both in basal conditions and after blocking the endogenous equilibrative nucleoside transporter activities using 10 μM dipyridamole.

Expression of the genes encoding CDKN1A/p21, TNFRF6/FAS, RRM2B, AQP3 and RPL3 was examined using real-time PCR, and changes in Bax protein were analyzed using Western blot (Fig. 4). 5′-DFUR treatment resulted in a dramatic up-regulation of all these genes, although the magnitude of the increase in mRNA levels was higher for FAS and AQP3, and to a lesser extent for p21, in MCF7–hCNT1 than in MCF7 cells. Moreover, under equilibrative transport inhibition conditions, the up-regulation of these genes was completely blocked in MCF7 cells, but unaltered in MCF7–hCNT1 cells (Fig. 4).

Finally, the effect of hCNT1 inhibition on the 5′-DFUR-induced transcriptomic response was analyzed using T-Ala at a concentration known to block hCNT1 function. MCF7–hCNT1 cells were treated for 90 min with 10 μM dipyridamole and 250 μM 5′-DFUR, either in the presence or in the absence of 100 μM T-Ala. Then, the mRNA levels of the genes encoding p21, FAS and AQP3 were examined at 24 h, using quantitative real-time PCR (Fig. 5). The increase in the mRNA levels triggered by 5′-DFUR was partially blocked by T-Ala in MCF7–hCNT1 cells, to a level expected from the known partial inhibition of hCNT1 function induced by T-Ala at this concentration. When the same experiment was performed using T-NH-Ala instead of T-Ala, the transcriptomic response to 5′-DFUR was not modified (not shown).

4. Discussion

Treatment of tumour cells with nucleoside-derived drugs activates the transcription of certain genes, most of which are involved in apoptosis and growth arrest [9,10]. Interestingly, short exposures to these drugs seem to be sufficient to exert the full transcriptomic response. In fact, 5′-DFUR treatment of MCF7 cells for periods as short as 90 min results in the up-regulation of at least 68 mRNAs, probably as the result of gene transcriptional activation, as deduced from a previous pharmacogenomic approach in which 9300 genes were analyzed [11]. This is consistent with the reported *in vivo* changes in gene expression profiles of breast cancer samples after 5-FU chemotherapy, resulting in the up-regulation of 23 genes [19]. In these conditions, it seems likely that transport processes play a key role in drug bioavailability and, accordingly, in the

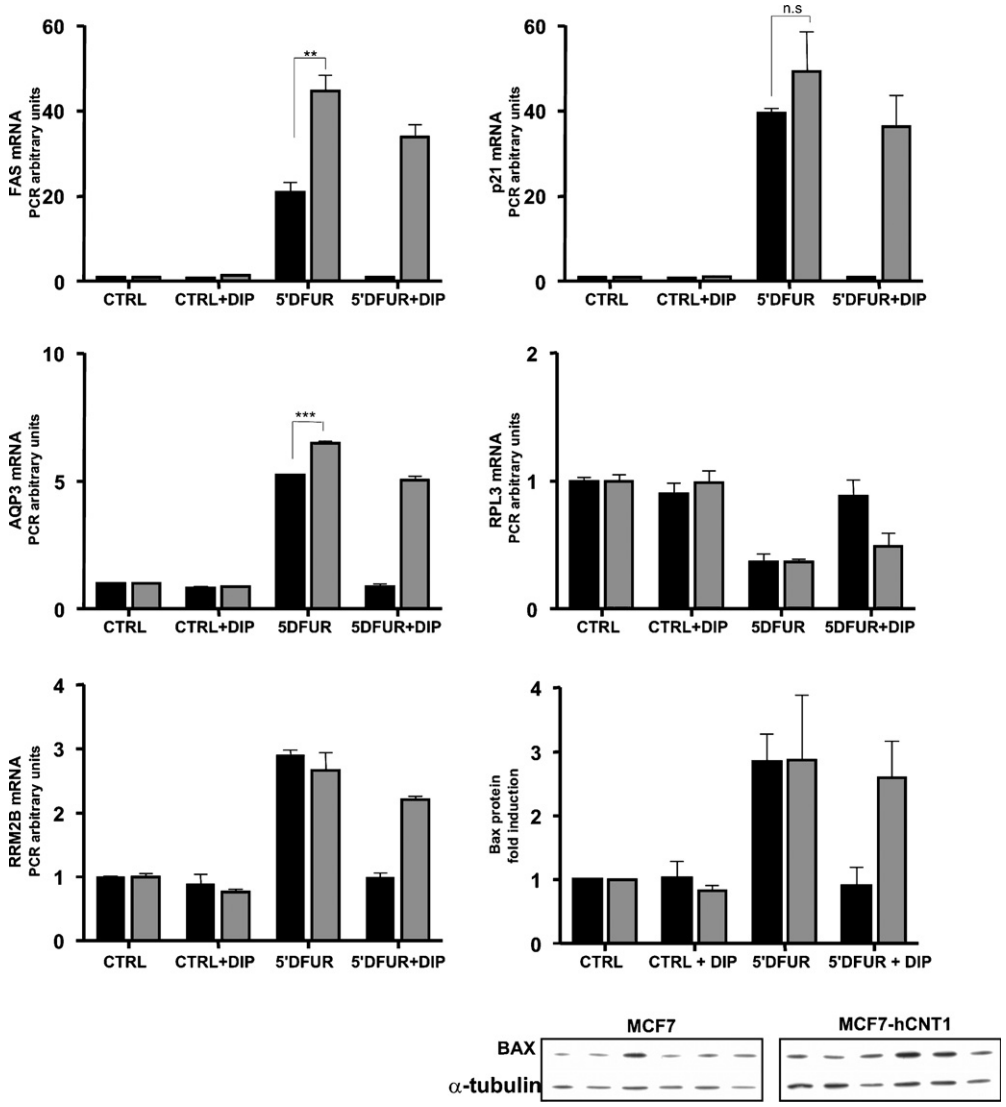

Fig. 4 – Induction of genes after 5′-DFUR treatment. Quantitative RT-PCR analysis for the genes encoding CDKN1A/p21, TNFRF6/FAS, RRM2B, AQP3 and RPL3, 24 h after a 90-min treatment with 250 μM 5′-DFUR in MCF7 (black) and hCNT1–MCF7 (grey) cells. C_T values for each gene have been normalized to an endogenous reference gene (GAPDH). Results are the mean ± S.E. of three experiments. Statistical significance was assessed using the Student's t test ($^{**}P < 0.01$, $^{***}P < 0.001$). Western blot analysis of Bax protein expression 24 h after a 90-min treatment with 250 μM 5′-DFUR in MCF7 and hCNT1–MCF7 cells. The induction triggered by treatment was quantified densitometrically. Results (mean ± S.E.M. of three independent experiments) are shown as arbitrary units normalized to α-tubulin.

transcriptomic response triggered by treatment. As we recently showed, inhibition of hENT1-related function by NBTI blocks the up-regulation of most genes activated by 5′-DFUR in MCF7 cells, even when hENT2 function remains unaltered, which highlights the relevance of specific transporter-mediated drug uptake in 5′-DFUR intracellular action [11]. Variability in hENT1 expression has been found in different human tumours (including some hENT1-negative cases) thus suggesting a complex pattern of NT expression in cancer [8,20–23]. We now provide compelling evidence for a ''chemotherapeutic rescue'' of 5′-DFUR-induced cytoxicity in MCF7 cells, under conditions of hENT1 and hENT2 pharmacological blockade, associated with the heterologous expression of hCNT1 protein. Human CNT1 expression also restores the up-regulation of a panel of mRNAs, selected among the series of genes regulated in response to 5′-DFUR, previously identified in MCF7 cells. This finding indicates that the pattern of nucleoside transporter expression in tumour cells rather than the occurrence of a particular NT isoform is responsible for the response to nucleoside-based chemotherapy.

The particular transporter protein (hCNT1) that rescues the 5′-DFUR-induced transcriptomic response of MCF7 cells, under conditions of hENT inhibition, is a high-affinity pyrimidine nucleoside-preferring transporter expressed in most epithelial cell types [24–26]. It can translocate a variety of nucleoside-derived anticancer drugs, such as 5′-DFUR [16].

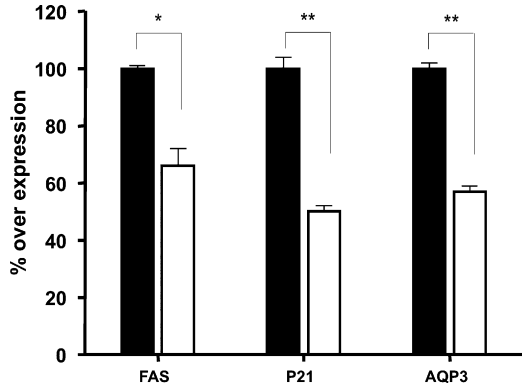

Fig. 5 – Inhibition of transcriptional targets of 5′-DFUR by T-Ala. Quantitative RT-PCR analysis for the genes encoding CDKN1A/p21, TNFRF6/FAS and AQP3. MCF7–hCNT1 cells were incubated for 90 min in the presence of 250 μM 5′-DFUR, 10 μM dipyridamole (closed) and 100 μM T-Ala (open). Results are the mean ± S.E.M. of three experiments. Statistical significance was assessed using the Student's t test ($^{*}P < 0.05$, $^{**}P < 0.01$).

Here we provide further evidence that hCNT1 expression can promote sensitization to this drug in the human breast cancer cell line, MCF7. Some genes (those encoding FAS, AQP3 and perhaps p21) are still further up-regulated by 5′-DFUR in MCF7–hCNT1 cells, which suggests that these genes may contribute to the further increase in chemosensitivity.

Although here it is demonstrated that hCNT1 confers sensitivity to nucleoside-derived drugs (5′-DFUR) in a human tumour cell line, it is important to stress that under conditions of hENT blockade, a situation in which cells become totally resistant to treatment, hCNT1 expression confers the same sensitivity and transcriptomic response as that observed in MCF7 and MCF7–hCNT1 cells, in which hENT1 and hENT2 proteins are functional. The complementary, or even compensatory role of hCNT1 in cases of hENT expression loss is also supported by data obtained under conditions of hCNT1 pharmacological inhibition. As discussed above, there are no suitable hCNT blockers, although we addressed this issue by synthesizing a 3′-OH modified pyrimidine nucleoside that allows partial inhibition of hCNT1 function, with a slight inhibition of the equilibrative transporters endogenously expressed in this particular cell model. Partial inhibition of hCNT1 function does prevent, in a similarly partial manner, hCNT1-induced cytotoxic and transcriptomic responses to 5′-DFUR. It is difficult to synthesize highly selective and specific CNT inhibitors since all SLC28- and SLC29-encoded proteins show overlapping substrate selectivity. Nevertheless, T-Ala and its inactive counterpart, T-NH-Ala, despite their limitations appear to be suitable molecules for the purpose of the present study. Here we have found a good correlation between the level of hCNT1 inhibition achieved and its impact on the cytotoxic and transcriptomic responses, which suggest that these effects are only accounted for by hCNT1 function. Moreover, compensatory effects of hCNT1 introduction into MCF7 cells on 5′DFUR cytotoxicity might apply to nucleoside-derived drugs in general, since similar results were obtained when using gemcitabine, even at longer incubation times (24 h) (not shown).

An additional issue regarding this study is whether or not restoring hCNT1 function in MCF7 cells is relevant from a physiological point of view. Human CNT1 expression is known to be highly dependent upon the differentiation status of the cell [27]. In fact, although MCF7 cells do not show significant hCNT1-related transport activity, its associated mRNA and protein could be easily detected. Moreover, MCF7–hCNT1 cells exhibit a huge increase in hCNT1 protein levels, although hCNT1-related activity was moderately increased (50%) above endogenous ENT-related activity. This may be explained either by the fact that the hCNT1 is not fully functional or by the more likely possibility that most of this protein expressed in MCF7 cells is localized intracellularly. In fact, hCNT1 localization is often associated with intracellular stores even in normal tissues and tumours [6,28]. Although we have recently identified physiological modulators of CNT2-regulated insertion into the plasma membrane [29], it is not yet known how hCNT1 protein trafficking into and from the plasma membrane occurs. However, in hepatocytic BC2 cells, hCNT1 activity can only be detected when polarization is achieved [27].

As well as the different localization of NTs within the cells, evidence for variability in transporter expression, including in some cases complete loss of expression, has also been recorded in recent years [12,22,23]. Moreover, co-expression and variability of different transporters has also been reported in gynaecologic tumours, in which hCNT1, hENT1 and hENT2 expression was assessed. Interestingly, hCNT1 loss associated with particular histological subtypes was characterized by poor prognosis [12]. Moreover, other clinical correlations between nucleoside transporters and tumour sensitivity to treatment have been reported [8,30,31]. Therefore, although the role of nucleoside transporters in nucleoside-derived drug sensitivity has been demonstrated in different studies, the co-expression of different transporters and their variability would suggest that it is the overall transporter expression pattern rather than the expression of an individual transporter that may better explain the response to nucleoside-derived drugs.

In summary, we have engineered breast cancer MCF7 cells to express the high-affinity pyrimidine-nucleoside transporter protein hCNT1, in order to determine whether this transporter can rescue the loss of nucleoside-derived drug cytotoxic activity, induced under conditions of hENT1 and hENT2 inhibition. We have addressed this issue both by monitoring the cytotoxic effect of the capecitabine intermediate 5′-DFUR on these cell lines and by analysing its effects on selected genes previously identified using a pharmacogenomic approach. These data are consistent with the idea that retention of hCNT1 expression alone might be sufficient for nucleoside-derived drugs to exert their cytotoxic actions in tumours, even if they have lost hENT expression and related activities. The results of this study thus highlight the relevance of the combined expression pattern of nucleoside transporters, notably hENT1 and hCNT1, in the response of patients to nucleoside-derived drug chemotherapy.

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