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EXPRESSION AND DISTRIBUTION OF NUCLEOSIDE TRANSPORTER PROTEINS IN THE HUMAN SYNCYTIOTROPHOBLAST

Ekaitz Errasti-Murugarren, Paula Díaz, Valeria Godoy, Gloria Riquelme and Marçal Pastor-Anglada

Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Spain [EE-M, VG, MP-A].

IBUB - Institut de Biomedicina de la Universitat de Barcelona, Spain [EE-M, VG, MP-A].

CIBER EHD, Spain [EE-M, MP-A].

Departamento de Fisiología y Biofísica. Instituto de Ciencias Biomédicas (ICBM), Facultad de Medicina, Universidad de Chile; Santiago, Chile [PD, VG, GR].

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Running title: Nucleoside transporters in human placenta

Corresponding author address: Marçal Pastor-Anglada, Ph.D. Departament de Bioquímica i Biologia Molecular. Institute of Biomedicine (IBUB), Universitat de Barcelona and CIBER EHD. Avda Diagonal 645. Edifici annex. Planta -1. 08028 Barcelona.

Tel. +34 93 402 1543 / 1544. Fax +34 93 402 1559. E-mail: mpastor@ub.edu

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Abbreviations List: hCNT, human concentrative nucleoside transporter; hENT, human equilibrative nucleoside transporter; PBS, phosphate buffered saline; 5´-DFUR, 5´-deoxy-5-fluorouridine.

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ABSTRACT

The plasma membrane distribution and related biological activity of nucleoside transporter proteins (NT) have been investigated in human syncytiotrophoblast from term placenta using a variety of approaches, including nucleoside uptake measurements into vesicles from selected plasma membrane domains, NT immunohistochemistry and subcellular localization (basal, heavy and light apical membranes as well as raft enriched membranes from the apical domain). Conversely to other epithelia, we have identified the high-affinity pyrimidine-preferring concentrative nucleoside transporter hCNT1 as the only hCNT-type protein expressed at both the basal and apical membranes of this epithelium. hCNT1 localization in lipid rafts is also dependent upon its subcellular localization in the apical plasma membrane, suggesting a complex cellular and regional expression. Overall, this favours the view that the placenta is a pyrimidine-preferring nucleoside sink from both maternal and fetal sides and hCNT1 plays a major role in promoting pyrimidine salvage and placental growth. This finding may be of pharmacological relevance, because hCNT1 is known to interact with anticancer nucleoside-derived drugs and other molecules, such as nicotine and caffeine, for which a great variety of harmful effects on placental and fetal development, including IUGR, have been reported.

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INTRODUCTION

Fetal exposure to xenobiotics is known to be harmful in many cases, compromising gestation itself and neonatal development. The placenta is known to express a broad scope of xenobiotic transporters (Leazer and Klaassen, 2003), which appear to be key players in the regulation of placental vectorial flux of nutrients and xenobiotics, either potentiating their materno-fetal transfer or their detoxification. Among the plasma membrane proteins that might play this dual role, being both nutrient and xenobiotic transporters, most members of the *SLC28* and *SLC29* gene families have been reported to be expressed in the placenta at the mRNA level (Barros et al., 1995; Errasti-Murugarren et al., 2009; Govindarajan et al., 2007; Gray et al., 2004; Griffiths et al., 1997). *SLC28* and *SLC29* genes encode human Concentrative Nucleoside Transporter (hCNT) and human Equilibrative Nucleoside Transporter (hENT) proteins, respectively (Pastor-Anglada et al., 2008; Young et al., 2008). To date, only human equilibrative nucleoside transporter proteins 1 and 2 (hENT1 and hENT2) have been reported to be expressed in placenta, although their plasma membrane localization is still unclear (Barros et al., 1995; Govindarajan et al., 2007). Occurrence of hCNT proteins in human placenta has not been studied in detail, although the human syncytiotrophoblast derived cell line BeWo has been shown to express hCNT3 mRNA and protein (Yamamoto et al., 2007).

The elucidation of what particular types of CNT proteins are expressed in the human syncytiotrophoblast, along with the analysis of their polarized expression relative to that of ENT type proteins may be of great interest in pharmacology. This is based upon the evidence that NT proteins mediate the translocation of natural

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nucleosides, but also the uptake of most nucleoside-derived anticancer drugs and some nucleoside-derived inhibitors of retroviral reverse transcriptases used in antiviral therapies (Errasti-Murugarren and Pastor-Anglada, 2010). Some reports have related nucleoside-derived therapies with placental dysfunction. The demethylating agent 5-azacytidine, a good hCNT1 and hENT1 substrate (Huang et al., 2004; Rius et al., 2009) has been reported to induce a variety of harmful effects in rodent placenta, including reduced mass, altered structure and impaired proliferation of trophoblast cells (Serman et al., 2007). Gemcitabine, a suitable hCNT1, hCNT3, hENT1 and hENT2 substrate (Errasti-Murugarren et al., 2007; Mackey et al., 1999), has been reported to induce developmental toxicity in mice (Eudaly et al., 1993) whereas it has been shown very recently that placental tissue of HIV-1 infected antiretroviral therapy (ART)-exposed pregnancies shows mitochondrial DNA depletion (Gingelmaier et al., 2009). Moreover, HIV-uninfected children born to HIV-infected mothers are apparently at risk of long-term mitochondrial toxicity (Poirier et al., 2003).

Interestingly, nucleoside transporters are also known to interact with compounds such as caffeine and nicotine with high affinity (Lang et al., 2004). Both xenobiotics have been comprehensively studied as risk factors during pregnancy. Among many other observations in the literature, it is known that caffeine intake increases the risk of early spontaneous abortion among non-smoking women (Cnattingius et al., 2000), whereas modest maternal caffeine exposure may impair fetal cardiovascular function and growth (Momoi et al., 2008) but also promotes long-lasting behavioural changes in mouse offspring (Bjorklund et al., 2008). Nicotine exposure during pregnancy has also been related to a variety of harmful effects, including, among many others, intrauterine growth retardation (IUGR) (Einarson and Riordan, 2009).

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Based upon this previous evidence, the goal of this contribution was to investigate the expression, activity, and subcellular localization of nucleoside transporters in non-pathological syncytiotrophoblast. This is of crucial interest for the understanding of the role these transporters play in placental physiology, but more importantly, this study also represents the first step in the future analysis of how drug-induced placental dysfunction can be associated with altered NT-related transport processes, thereby affecting fetal growth and performance.

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MATERIALS AND METHODS

Reagents: Uridine, cytidine, adenosine, thymidine, guanosine, 5´-deoxy-5-fluorouridine and zidovudine (AZT) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Tritiated uridine ([5,6-^{3}H], 35-50 Ci/mmol), thymidine ([5,6-^{3}H], 35-50 Ci/mmol), and adenosine ([5,6-^{3}H], 35-50 Ci/mmol) were purchased from Amersham Biosciences (Buckinghamshire, UK). Cytidine ([5-^{3}H(N)], 21.5 Ci/mmol), and guanosine ([8-^{3}H(N)], 7 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA, USA).

RNA isolation and RT-PCR reaction: Total RNA was extracted from placenta lysates using an RNeasy Mini Kit (Qiagen, Barcelona, Spain). RNA was treated with DNase I from an RNase Free DNase Set (Qiagen, Barcelona, Spain) to eliminate contaminating DNA. cDNA was generated from 1 μg total RNA by reverse transcription using TaqMan reagents as described by the manufacturer (Applied Biosystems, Foster City, CA, USA). Different sets of primers were designed and synthesized for PCR analysis of each transporter. For amplifying hCNT1, the primers used were 5´-GAG GGG TCT AGC TCT TGC TG-3´ (forward) and 5´-CAC CTT CAC GGA GAT GGC GGC C-3´ (reverse), which generated a 822-bp product; for hCNT2, the primers were 5´-CCC GCC TGA GGC TTT GGA CG-3´ and 5´-CAA CCC CAA AGG CTA TGA AGG-3´, which generated a 650-bp product; for hCNT3, the primers were 5´-GCA CAC TCA AAC TGC TCC ACC-3´ and 5´-GGG CTC TGT GAA AGT TCA GC-3´, which generated a 446-bp product and a 622-bp insert-containing (hCNT3ins) product; for hENT1, the primers were 5´-GGG CCA CCG CCT GCT GAA ACG G-3´ and 5´-CCT TGA CCA GCG CCT CCC C-3´, which generated a 410-bp

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product; and for hENT2, the primers were 5´-CCT CAA CTC CTT CCT GTA CC-3´ and 5´-CCC ACA CAG GGC GTG ATA AAG-3´, which generated a 355-bp product. PCR reactions were carried out using the following cycling conditions: 40 cycles of 94ºC for 1 min; 55ºC (hCNT1, hCNT2, hENT1 and hENT2) or 57ºC (hCNT3) for 1 min and 72ºC for 3 min, followed by a final extension at 72ºC for 15 min and cooling to 4ºC. The amplified fragments were separated and visualized on 1% ethidium bromide-stained agarose gels.

Preparation of syncytiotrophoblast plasma membranes: Placentae were obtained from normal pregnancies and collected at term immediately after delivery from the San José Hospital Maternity Unit (Santiago, Chile). Human placental plasma membrane vesicles were prepared from fresh normal-term placentae by a previously described method that allows the simultaneous isolation of apical and basal membranes from the same placenta (Jimenez et al., 2004). This method involves differential centrifugation, precipitation of non-microvillous membranes with magnesium ions and a sucrose gradient step. All solutions were buffered with 20 mM Tris-Maleate, pH 7.4. A portion (0.5-1 ml) of either the microvillous or basal membrane-enriched fraction was overlaid on the surface of a discontinuous sucrose gradient. The bands at the 10%/37% and 37%/45% sucrose interfaces correspond to the light microvillous membrane fraction (LMVM) and heavy microvillous membrane fraction (MVM), respectively, whereas the band at the 47%/52% interface corresponds to the basal membrane fraction (BM). Each fraction was collected and diluted 10-fold with 20 mM Tris-Maleate (pH 7.4) and centrifuged at 110 000 x g for 30 min. The final pellets were resuspended in preload media containing 250 mM sucrose, 0.2 mM $CaCl_2$, 20 µM $MgCl_2$, 10 mM HEPES pH 7.5 and 150 mM KSCN and stored in liquid nitrogen. Protein concentration

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was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Inc. Rockford, IL, USA). The purity and enrichment of the apical and basal membrane fraction was determined routinely by assaying for classical marker protein activities as described (Jimenez et al., 2004). Alkaline phosphatase was used as an apical membrane marker. Enrichment of alkaline phosphatase activity was over 20-fold for MVM and LMVM when compared with the activity of the homogenate. The cross contamination of purified basal membranes with apical membranes was low, as evidenced by the lack of alkaline phosphatase enrichment in the basal fraction. The ratio of PLAP activity enrichment of BM compared to apical membranes, was 0.1 for the placentae used in this study, lower or equivalent to that from several other reports for single or paired apical and basal membrane preparations (Glazier et al., 1990; Illsley et al., 1990) as we demonstrated (Jimenez et al., 2004). Additionally, for both MVM and BM vesicles, orientation is preferentially right-side out, as reported previously (Illsley et al., 1990). For LMVM vesicles, data about orientation are not available. However, it is expected that following this isolation protocol, all three type of vesicles show a preferential right-side out orientation. In fact, when setting up these methods it was initially observed that preparations including both MVM and LMVM fractions showed at least 90% of properly oriented vesicles, as determined by measuring phosphatase alkaline activity before and after saponin treatment (Dr. Felipe Barros, Ph.D. Thesis 1993, unpublished), suggesting a preferential right-side out orientation for LMVM vesicles.

Nucleoside transport assay: Uptake studies in syncytiotrophoblast-derived plasma membrane vesicles were performed using the rapid filtration technique, as described previously (Ruiz-Montasell et al., 1992). Briefly, uptake was initiated by

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mixing 10 µl vesicle suspension with 40 µl uptake medium (250 mM sucrose, 0.2 mM $CaCl_2$, 20 µM $MgCl_2$, 10 mM HEPES, pH 7.5) containing either 150 mM NaSCN or KSCN and 1 µM [^{3}H]-labeled substrate. Reactions were terminated at the indicated times by adding 1 ml ice-cold stop solution (250 mM sucrose, 150 mM NaCl, 0.2 mM $CaCl_2$, 10 mM HEPES pH 7.5), and then filtered through 0.45-µm nitrocellulose filters. Filters were washed with 4 ml ice-cold stop solution and radioactivity on filters was determined subsequently by liquid scintillation counting. All experimental values were corrected by substracting non-mediated uptake values obtained by adding the stop solution into the transport before the vesicles. Additionally, to distinguish between hENT1 and hENT2 activities 1 µM nitrobenzylthioinosine (NBTI), a specific ENT1 inhibitor was added to the transport medium.

Preparation of apical lipid microdomains: Apical plasma membrane microdomains were isolated from MVM- and LMVM-enriched preparations as a separate detergent-resistant fraction by extracting with Triton X-100, as described by Godoy and Riquelme (Godoy and Riquelme, 2008). Briefly, aliquots (0.6 mg) of both isolated membrane fractions were homogenized 30 times in a manual glass homogenizer with 1% Triton X-100 in MBS-buffered saline (25 mM morpholinoethanesulphonic acid pH 6.5, 150 mM NaCl). After incubating for 90 min on ice, vesicles (1 ml) from both apical preparations (LMVM and MVM) were mixed with 1 ml 80% sucrose to obtain a final sucrose concentration of 40%. A discontinuous gradient was then prepared by overlaying the 40% cushion successively with 2 ml 35% sucrose and 1 ml 5% sucrose, and then tubes were centrifuged at 21 700 x g for 20-22 h in an AH-650 Sorvall swinging-bucket rotor (Wilmington, DE, USA). After

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centrifugation, 10 fractions (0.5 ml each) were collected starting at the top of the gradient.

Immunohistochemistry: Samples of human placental tissue from normal pregnancies were rinsed in 0.9% NaCl, frozen in cryogel, and sectioned. Sections (10 µm-thick) were fixed with 4% *p*-formaldehyde for 30 min and rinsed three times with Tris-buffered saline pH 7.6 (TBS) for 5 min. In parallel, 3.7% buffered formalin-fixed placentae were processed and paraffin embedded according to standard protocols. The 5-µm sections thus obtained were subsequently deparaffinized and rehydrated. Slides containing both types of tissue sections (fixed and paraffin-embedded) were treated with 0.1 M citrate buffer, pH 6.0 (antigen recovery treatment) and then incubated with 4% BSA for 1 h to block nonspecific binding. Slides were incubated overnight at 4ºC in a humidified chamber with primary polyclonal antibodies (diluted 1:100) against hENT1, hENT2 or hCNT1 (Farre et al., 2004). Slides stained for individual transporters were co-stained with mouse anti-cytokeratin 7 antibody (clone OVTL 12/30, Zymed Laboratories Inc.) diluted 1:100 in phosphate buffered saline, pH 7.4 (PBS), to confirm the trophoblastic localization of nucleoside transporters. After rinsing with PBS, tissue sections were incubated for 1 h at room temperature with Rhodamine Red™ X-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch, Code Number 111-295-003; 1:200), which recognizes anti-hENT1, anti-hENT2 and anti-hCNT1), and with Cy™ 2-conjugated goat anti-mouse antibody (Jackson Immunoresearch, Code Number 115-225-003), which recognizes the anti-cytokeratin 7 antibody. Control sections were incubated with secondary antibody after incubation in PBS buffer without the primary antibody. Sections were viewed using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems Heidelberg GmbH, Manheim,

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Germany) equipped with a DMI6000 inverted microscope, a diode-pumped solid-state argon laser (561 nm) and a 63x oil-immersion objective (NA 1.4). Cy™ 2 and Red™ X labeling was visualized by acquiring images sequentially at the 488 and 561 nm laser lines using an Acousto-Optical Beam Splitter, with emission detection at 500-550 and 571-650 nm, respectively. Optical sections were collected every 0.3 microns in a 1024 x 1024 format with a confocal pinhole (radius) of 1 airy unit, zoom = 4 and a pixel size of 60 x 60 nm. Differential interference contrast images were acquired simultaneously.

Western blot analysis: All flotation gradient fractions were evaluated by SDS-PAGE and immunoblotting. An aliquot (50 µl) of each fraction was incubated with 10% (v/v) trichloroacetic acid (TCA) for 30 min on ice and centrifuged at 21 000 x g for 30 min at 4º. The pellets were resuspended in sample buffer and sonicated for 30 min. TCA-precipitated protein (40 µg) from LMVM and MVM preparations was separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad 162-0115). Membranes were incubated with primary anti-hENT1, anti-hENT2 or anti-hCNT1 antibodies diluted 1:1000. Also anti-human Placental Alkaline Phosphatase (anti-PLAP; clone 8B6; Sigma-Aldrich Inc.; 1:1000) and anti-human Transferrin Receptor (anti-htf-R; clone H68.4; Zymed Laboratories Inc.; 1:500) were used as raft and non-raft markers respectively. Following incubation with primary antibody, proteins were detected using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence (ECL) detection kit (Amersham, Buckinghamshire, UK).

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Data Analysis: Data are expressed as the mean ± S.D. of uptake values obtained in three filter inserts from three independent placentae. Data are representative of three experiments carried out on different days on different batches of membranes.

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RESULTS

A study of nucleoside transporter-related mRNA showed that analyzed five nucleoside transporters (hCNT1, hCNT2, hCNT3, hENT1 and hENT2), as well as the recently characterized spliced isoform of hCNT3 (hCNT3ins), were expressed in human placenta (Figure 1).

Nucleoside transport in syncytiotrophoblast plasma membrane-derived vesicles was characterized using either uridine or cytidine as substrates. Figure 2 shows the time course of uridine (A) and cytidine (B) uptake (1 μM) by vesicles derived from human placental LMVM (upper), MVM (middle) and BM (lower) (n = 3 independent placentae). Both sodium-dependent and independent components were observed. Inhibition of sodium-independent uptake with nitrobenzylthioinosine (NBTI) revealed that both NBTI-sensitive and -insensitive carriers, hENT1 and hENT2, respectively, appear functional in these membrane preparations (Figure 2), in agreement with the pattern of mRNA expression detected in this tissue (Figure 1). Sodium-dependent transport of both nucleosides into plasma membrane vesicles was observed when uptake was measured in the presence of an outside-to-inside sodium gradient (150 mM, out > in), similar to that reported in renal and intestinal brush-border membrane vesicles (Gutierrez and Giacomini, 1993; Ngo et al., 2001). Because nucleoside uptake was linear up to at least 40 s and this time point provided greater sensitivity, values obtained at 40 s are reported for all experiments conducted with placental syncytiotrophoblast plasma membrane vesicles. To analyze to which transport agency this sodium-dependent component was attributable the profiles of [^{3}H]-labeled natural nucleoside uptake in LMVM, MVM and BM vesicles and inhibition of [^{3}H]-uridine uptake by

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nucleosides and nucleoside-derived drugs were analyzed (Figure 3). Measurements of [^{3}H]-uridine, [^{3}H]-cytidine, [^{3}H]-thymidine, [^{3}H]-adenosine and [^{3}H]-guanosine uptake in the presence of a sodium gradient demonstrated the existence of sodium-dependent transport for all three pyrimidine nucleosides in both apical and basal membrane vesicles (Figure 3A). Sodium-coupled adenosine uptake in LMVM and MVM vesicles, though small, was significant, whereas sodium-dependent guanosine uptake was negligible. Moreover, sodium-dependent uptake of [^{3}H]-uridine (1 μM) was completely inhibited by 100 μM cytidine, thymidine and adenosine, but was not significantly blocked by 100 μM guanosine (n = 3 independent placentae) (Figure 3B). The nucleoside-derivatives, 5´-deoxy-5-fluorouridine and gemcitabine (100 μM), also significantly inhibited [^{3}H]-uridine uptake into apical and basal membrane vesicles, as did the nucleoside analogue zidovudine, albeit to a lesser extent (Figure 3B). All these results indicate that hCNT1 is the unique concentrative nucleoside transport activity present in syncytiotrophoblast plasma membrane-derived vesicles.

The immunohistochemical analysis of human placental tissue sections showed that hCNT1, hENT1 and hENT2 were expressed in the maternal-facing side of the syncytiotrophoblast (Figure 4, left). Although some immunofluorescence was evident in intracellular compartments, especially in some nuclei, staining was clearly most apparent at the syncytiotrophoblast apical membrane. Increasing exposure to the laser source revealed light staining of hENT2 and hCNT1 in the basal membranes, but basal membrane staining of hENT1 was not detected under any laser-exposure condition tested (data not shown). Double immunostaining with cytokeratin 7 confirmed the localization of these transporters to microvillous and basal syncytiotrophoblast membranes, demonstrating plasma membrane localization of nucleoside transporter

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proteins (Figure 4, right). No significant fluorescence was observed in slides treated with secondary antibodies only (data not shown).

The presence of distinct lipid rafts in the apical plasma membrane domain of placental syncytiotrophoblast has been recently reported (Godoy and Riquelme, 2008), although the presence of nutrient transporters, particularly nucleoside transporters, in these domains has not been established. In this regard, Western blot detection of nucleoside transporter proteins in purified syncytiotrophoblast membranes and analysis of the distribution of hCNT1, hENT1 and hENT2 in different lipid microdomains were carried out. Nucleoside transporter proteins in paired samples of purified apical and basal fractions from four placentae were analyzed by Western blotting. Figure 5A shows a representative Western blot with bands corresponding to the molecular weight of hCNT1 (upper), hENT1 (middle) and hENT2 (lower) in both LMVM and MVM apical membrane preparations as well as BM preparations. In agreement with uptake assay and immunohistochemical results, hCNT1 and hENT2 were present in all apical and basal purified fractions, whereas hENT1 was present only in light and heavy apical membranes.

To determine whether hCNT1, hENT1 and hENT2 are localized to membrane rafts, we isolated a detergent-resistant membrane fraction by sucrose density centrifugation, as recently described (Godoy and Riquelme, 2008). In Western blots of LMVM-containing gradient fractions (Figure 5B), hCNT1 and hENT1 immunoreactivity was detected in both detergent-resistant (lipid raft) and non-resistant fractions, although hCNT1 mainly partitioned into lipid raft fractions and hENT1 was predominantly associated with non-lipid raft fractions. In contrast, hENT2 was totally

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absent from the detergent-resistant membranes of the LMVM flotation fractions, indicating that hENT2 does not localize to lipid rafts in this fraction. Western blotting of gradient fractions enriched for MVM showed a completely different distribution of both hCNT1 and hENT1 transporter proteins. Here, both transporters were absent from the low sucrose density fractions, and were found exclusively in non-raft fractions. hENT2 immunoreactivity was found mainly in non-raft fractions, although it could be detected in some, but not all, lipid raft fractions (Figure 5B).

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DISCUSSION

Materno-fetal transfer of nutrients like glucose and amino acids is mostly determined by the activity of specific transporter proteins expressed in the placental syncytiotrophoblast plasma membranes. Glucose transport from the mother to the fetus appears to be influenced by the maternal blood glucose concentration across the placenta and is mediated by members of the facilitative GLUT transporters (Baumann et al., 2002), although evidence for SLGT1-mediated Na-coupled transport in the syncytiotrophoblast has also been provided (Kevorkova et al., 2007). The materno-fetal transfer of most amino acids is dependent on the coupling of secondary active amino acid transporters localized in the microvillous apical membrane of the syncytiotrophoblast (in direct contact with the maternal blood) and facilitative transporters present in the basal membrane (facing the fetal circulation) (Grillo et al., 2008). This model of nutrient vectorial flux is to some extent similar to the one described for nucleosides in (re)absorptive epithelia (intestinal and renal epithelial cells), also involving the asymmetric distribution of concentrative (CNT-type) and equilibrative (ENT-type) nucleoside transporter proteins in apical and basolateral domains, respectively, although some ENT1 expression at the apical side has also been reported (Errasti-Murugarren et al., 2007).

Interestingly, the “absorptive model” for the vectorial flux of nucleosides is not the only one that could be applied to the syncytiotrophoblast epithelium. Previous evidence supported an apical and basal localization of equilibrative nucleoside transport activities and transporters in the syncytiotrophoblast (Barros et al., 1995; Govindarajan et al., 2007). This is further reinforced in this contribution by combining functional data

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and hENT1 and hENT2 immunodetection. hENT2 was present in both domains (apical and basal) when determined by Western blot, although this pattern was not exactly reproduced when looking at hENT2 expression by immunohistochemistry. Putative post–translational modifications of the protein, maybe related to its polarized sorting, might interfere with staining procedures. Regarding hENT1, its apical localization was corroborated by Western blot experiments. However, in agreement with previous observations (Barros et al., 1995; Govindarajan et al., 2007), a hENT1-like activity identified at the basal plasma membrane vesicles was not associated with a detectable hENT1 protein, as measured by either Western-blot or immunohistochemistry. The possibility of two distinct isoforms of the hENT1 protein in the syncytiotrophoblast apical and basal membranes should be further evaluated. In fact, splicing isoforms of the *SLC29A1* gene could affect both protein immunodetection and/or subcellular localization in polarized epithelia as previously reported for the *SLC28A3* gene product (Errasti-Murugarren et al., 2009). Alternatively, there is the possibility that immunoreactivity using antibodies raised against the intracellular loop between transmembrane domains 6 and 7 might be impaired if significant post-translational modification occurs in the loop (i.e. phosphorylation). In any case, it appears that transport activities consistent with the occurrence of both types of transporter proteins (hENT1 and hENT2) are present at both poles of this epithelium.

Nevertheless and more importantly, this study also demonstrates that a concentrative nucleoside transport activity, mostly if not exclusively linked to the expression of the pyrimidine-preferring nucleoside transporter hCNT1, is found at both the microvillous and basal membranes of the syncytiotrophoblast. This represents a unique distribution of CNT-type proteins in a polarized epithelium. The occurrence of

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hCNT1 is based upon the selectivity profile of the uptake of natural nucleosides and the *cis*-inhibition of uridine uptake by nucleosides into apical and basal plasma membrane vesicles. In this case, the functional evidence is strongly supported by hCNT1 immunodetection. In fact, immunohistochemical analysis of placental sections further identified hCNT1 at both poles of the syncytiotrophoblast. The low staining for hCNT1 at the basal side is probably due to its comparatively low expression in this membrane domain, as confirmed by the activity measurements and the Western blot analysis of the subcellular membrane fractions.

All these observations, taken together would be consistent with the model of NT protein distribution in the syncytiotrophoblast shown in Figure 6, a model which rules out the occurrence of hCNT2/3 functional expression, at least in placentae at term. Moreover, residual adenosine uptake found in vesicles would be consistent with previous evidence showing that adenosine is a high-affinity hCNT1 inhibitor (with a K_i value in the low micromolar range) but a poor permeant (Larrayoz et al., 2004; Smith et al., 2004), suggesting that endogenous maternal adenosine levels could reduce interaction of hCNT1 with nucleoside-derived drugs, reducing in that way their fetal toxicity. In agreement with previous observations (Yamamoto et al., 2007), all five NT-related mRNAs could be found in placenta. This is consistent with either CNT2/3 transporters being expressed in other cell types or being developmentally regulated in the syncytiotrophoblast, or both. Nevertheless, the occurrence of hCNT1 as the unique concentrative nucleoside transporter in this epithelium has a physiological rationale. The expression levels (mRNA transcript amounts) of the enzymes implicated in both pyrimidine and purine nucleoside metabolism in placenta, have been searched in the Unigene Database (Sayers et al., 2009). The enzyme machinery expressed in placenta is

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consistent with high purine but low pyrimidine nucleotide *de novo* synthesis. Interestingly, the enzyme machinery implicated in salvage pathways shows the opposite pattern, being the enzymes responsible for pyrimidine recycling highly expressed. This scenario is consistent with the need of a plasma membrane pyrimidine-preferring concentrative nucleoside transporter (hCNT1) to mediate pyrimidine, but not purine, salvage.

It is not evident whether this NT distribution pattern will in turn confer vectoriality to nucleoside transport across the placenta. The fetus is highly dependent on glucose and amino acids, which are the building blocks of purine and pyrimidine nucleotides and *de novo* synthesis is highly prevalent in the growing fetus (Alexiou and Leese, 1992; Boza et al., 1996). In this context, it is tempting to speculate that both apical and basal hCNT1 are implicated in the pyrimidine nucleoside supply to the placenta, from both maternal and fetal sides. This would be consistent also with the expression pattern of hENT2, present at both poles, being the only NT protein known to mediate transport of nucleobases, which could also be used for pyrimidine salvage purposes (Figure 6). As discussed above, this model is based upon results obtained in term placenta. A previous report showed slight variations in thymidine uptake (a hCNT1 substrate) along different gestational stages (Sooranna et al., 1999). In any case this was total uptake and does not rule out the possibility that the contribution of particular nucleoside transporter proteins to nucleoside uptake changes during placental growth. This may be of interest in the understanding of placental physiology but difficult to address in human studies.

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As introduced above, hCNT1 is a high affinity transporter for selected anticancer drugs, such as gemcitabine and 5-azacytidine (Garcia-Manteiga et al., 2003; Rius et al., 2009). These drugs have been reported to induce a variety of harmful effects in rodent placenta and developmental toxicity as well (Eudaly et al., 1993; Serman et al., 2007). Moreover, inhibition of hCNT1 by selected drugs may compromise pyrimidine salvage by the syncytiotrophoblast thus contributing to impaired placental function. Conclusions drawn from the use of cell lines expressing exclusively low affinity ENT-type transporters should be taken cautiously and re-evaluated (Chishu et al., 2008).

Finally, the occurrence of selected hNT proteins in raft microdomains might be relevant also in functional terms. We have recently shown that localization of hCNT3 in lipid-rafts might determine its biological activity (Errasti-Murugarren et al., 2010), and interestingly, CNT1 protein had also been identified in a caveolin-enriched plasma membrane fraction isolated from quiescent rat liver (Duflot et al., 2002). Based upon this previous evidence the analysis of the localization of hNT proteins in lipid rafts was performed. As described above, Western blotting of the LMVM gradient showed a bimodal distribution for hCNT1 and hENT1 immunoreactivities, being detected both in lipid raft and non-lipid raft fractions, whereas hENT2 was totally absent in raft fractions. Interestingly, the heavy apical microvillous membrane (MVM) showed a completely different distribution for both hCNT1 and hENT1 transporter proteins, being found exclusively in non-raft fractions. The heavy (MVM) and light (LMVM) microvillous membrane fractions used in this study correspond to the finger-like projections and the bottom part between the microvilli, respectively (Godoy and Riquelme, 2008), which additionally show a heterogeneous population of raft domains (Braccia et al., 2003; Godoy and Riquelme, 2008). The expression of particular

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cytoskeletal proteins in finger-like projection regions of the microvilli (MVM) that has been proposed to be tightly associated with plasma membrane lipidic microdomains could explain the presence of lipid-raft markers in non-raft fractions (Godoy and Riquelme, 2008). In this regard, both hCNT1 and hENT1 transporters in MVM are located mainly in non-raft fractions which are clearly defined by the human transferrin receptor, whereas distribution of hENT2 protein is less clear. All these observations, taking together, suggest that nucleoside transporter proteins do not only show heterogeneous distribution in polarized epithelia but also at the subcellular level, in particular apical domains. Although the elucidation of the physiological rationale for this distribution and the effect on nucleoside transport activity requires further analysis, it is interesting to point out that preliminary data from our laboratories (unpublished observations) anticipate that in some pathologies, like pre-eclampsia, disruption of lipid raft domains will result in altered distribution of hNT proteins thus probably affecting their biological function.

In summary, this study provides the first comprehensive analysis of nucleoside transporter expression in human syncytiotrophoblast. The only concentrative nucleoside transporter protein expressed at significant levels in this epithelium at term is hCNT1, a pyrimidine preferring, sodium coupled concentrative nucleoside transporter. Its occurrence at both poles of the epithelium, basal and apical, is consistent with the fact that the placenta has the ability to synthesize purine nucleotides *de novo* but appears to be dependent on pyrimidine nucleoside salvage. Thus, hCNT1 would be a key player in this process. Considering that some anticancer nucleoside-derived drugs and xenobiotics such as nicotine and caffeine are either translocated by or bound to hCNT1 with high affinity, we suggest that most of the harmful effects triggered by these drugs

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can be mediated, at least in part, by its ability to interact with hCNT1 thus impairing pyrimidine nucleoside-salvage process.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Errasti-Murugarren E., Pastor-Anglada M. and Riquelme G.

Performed experiments: Errasti-Murugarren E., Díaz P. and Godoy V.

Performed data analysis: Errasti-Murugarren E., Díaz P., Pastor-Anglada M. and Riquelme G.

Wrote or contributed to the writing of the manuscript: Errasti-Murugarren E., Díaz P., Pastor-Anglada M. and Riquelme G.

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FOOTNOTES

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The laboratories of G. Riquelme and M. Pastor-Anglada contributed equally to this work.

EE-M present address:

Institut de Recerca Biomèdica (IRB), Barcelona, Spain

PD present address:

Maternal and Fetal Health Research Centre, University of Manchester, Manchester, United Kingdom

Please send reprint requests to:

Marçal Pastor-Anglada, Ph.D. Departament de Bioquímica i Biologia Molecular. Institut de Biomedicina (IBUB), Universitat de Barcelona and CIBER EHD. Avda Diagonal 645. Edifici annex. Planta -1. 08028 Barcelona.

Tlf. +34 93 402 1543 / 1544.

Fax +34 93 402 1559.

E-mail: mpastor@ub.edu

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FIGURE LEGENDS

Figure 1. Qualitative RT-PCR analysis of nucleoside transporters in human placenta. RT-PCR was used to amplify human concentrative nucleoside transporters 1, 2 and 3 (hCNT1, hCNT2 and hCNT3, upper) and equilibrative nucleoside transporters 1 and 2 (hENT1 and hENT2, lower) from human placenta (P). Kidney (K), liver (L) and pancreas (Pc) were used as positive controls for hCNT1, 2 and 3, respectively and kidney for both hENTs. Positive controls for both wild type (446-bp) and the recently reported hCNT3 splicing isoform hCNT3ins (622-bp) are shown. Representative agarose gel showing the amplification of cDNA fragments of the anticipated size is shown. C-: Negative control.

Figure 2. Time course of nucleoside uptake. Time course of [^{3}H]-uridine (A) and [^{3}H]-cytidine (B) (1 μM) uptake in syncytiotrophoblast LMVM (upper), MVM (middle) and BM (lower) vesicles. Uptake into vesicles was assayed in medium containing radioactive uridine or cytidine and either 150 mM sodium (NaSCN, ■) or potassium (KSCN, ▲) as described in *Materials and Methods*. Equilibrative nucleoside transport activities were identified by means of NBTI (1 μM) inhibition (▼). Results are presented as mean ± S.D. of triplicate estimations made on three independent preparations.

Figure 3. Characterization of CNT activity in plasma membrane vesicles. (A) Sodium-dependent uptake of pyrimidine and purine nucleosides (1 μM) by LMVM, MVM and BM at 40 s was measured in transport medium containing 150 mM NaSCN or 150 mM KSCN. Sodium-dependent transport was calculated as uptake in sodium

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medium minus uptake in potassium medium. (B) Inhibition profile of sodium-dependent [^{3}H]-uridine uptake (1 μM) by LMVM, MVM and BM at 40 s. Data are presented as percentage of uridine transport normalized to the control. Transport experiments in (A) and (B) were carried out as in Figure 2. Data are expressed as the mean ± S.D. of triplicate estimations made on three independent preparations.

Figure 4. Expression of hCNT1, hENT1 and hENT2 proteins in human term placenta. Confocal fluorescence micrograph of immunohistochemical sections of a normal-term human placenta showing hCNT1 (A), hENT1 (B) and hENT2 (C) staining of the apical syncytiotrophoblast membrane. Double immunostaining with an anti-cytokeratin 7 antibody (D, E and F) shows intense staining of all three transporter proteins at the apical microvillous membrane of the syncytiotrophoblast (MVM) and in intracellular structures (G, H and I).

Figure 5. Distribution of nucleoside transporters in raft and non-raft fractions isolated from LMVM and MVM preparations. Lipid rafts were isolated from apical membrane vesicles, prepared from normal-term human placentae, by solubilizing in ice-cold 1% Triton X-100 and fractionating on a sucrose gradient. (A) Immunoblot analysis shows that both hCNT1 and hENT2 are present in apical and basal purified membrane-derived vesicles, whereas hENT1 is present only in apical membrane vesicles. (B) LMVM and MVM sucrose gradient fractions were separated by SDS-PAGE and analyzed by Western blotting using antibodies against hCNT1, hENT1 and hENT2, with placental alkaline phosphatase (PLAP) and human transferrin receptor (htf-R), used as raft and non-raft markers, respectively. *Upper panel:* A representative Western blot of LMVM samples showing the distribution of hCNT1 and hENT1 into both lipid

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raft and non-raft fractions, and exclusive distribution of hENT2 to non-raft fractions. *Lower panel:* Western blot of MVM samples showing localization of hCNT1 and hENT1 to non-raft fractions, and distribution of hENT2 to some raft and all non-raft fractions.

Figure 6. Model of nucleoside transporter protein distribution in the syncytiotrophoblast. Integrated model of nucleoside, amino acid and glucose transporters distribution in human placenta together with expression levels of nucleoside metabolism key enzymes. Abbreviations: PRPS1/2: Phosphoribosyl pyrophosphate synthase 1/2; CAD: Carbamoyl phosphate synthetase II/ aspartate transcarbamoylase/ dihydroorotase; DD: Dihydroorotate dehydrogenase; UP: Uridine phosphorylase 1; UCK: Uridine-cytidine kinase; TK: Thymidine kinase; TP: Thymidine phosphorylase; PyN: Pyrimidine nucleoside; PN: Purine nucleoside; NB: Nucleobase; NP; Nucleoside phosphate; AAT: Amino acid transporter.

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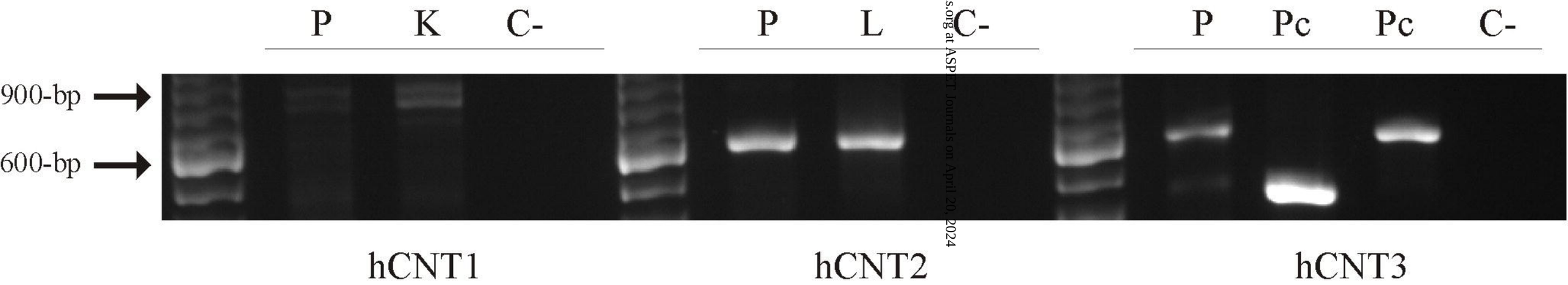
Figure 1



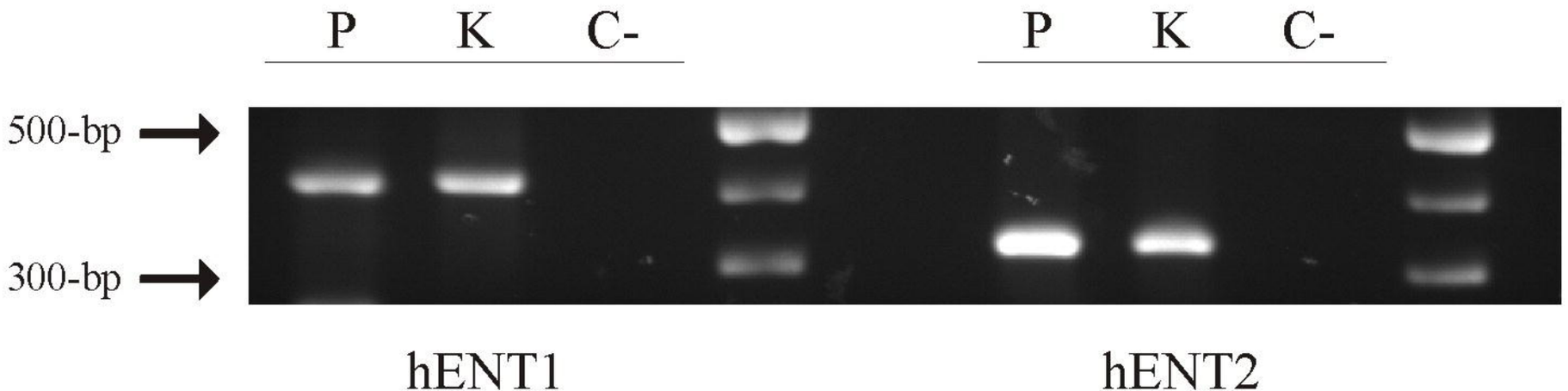


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Figure 2

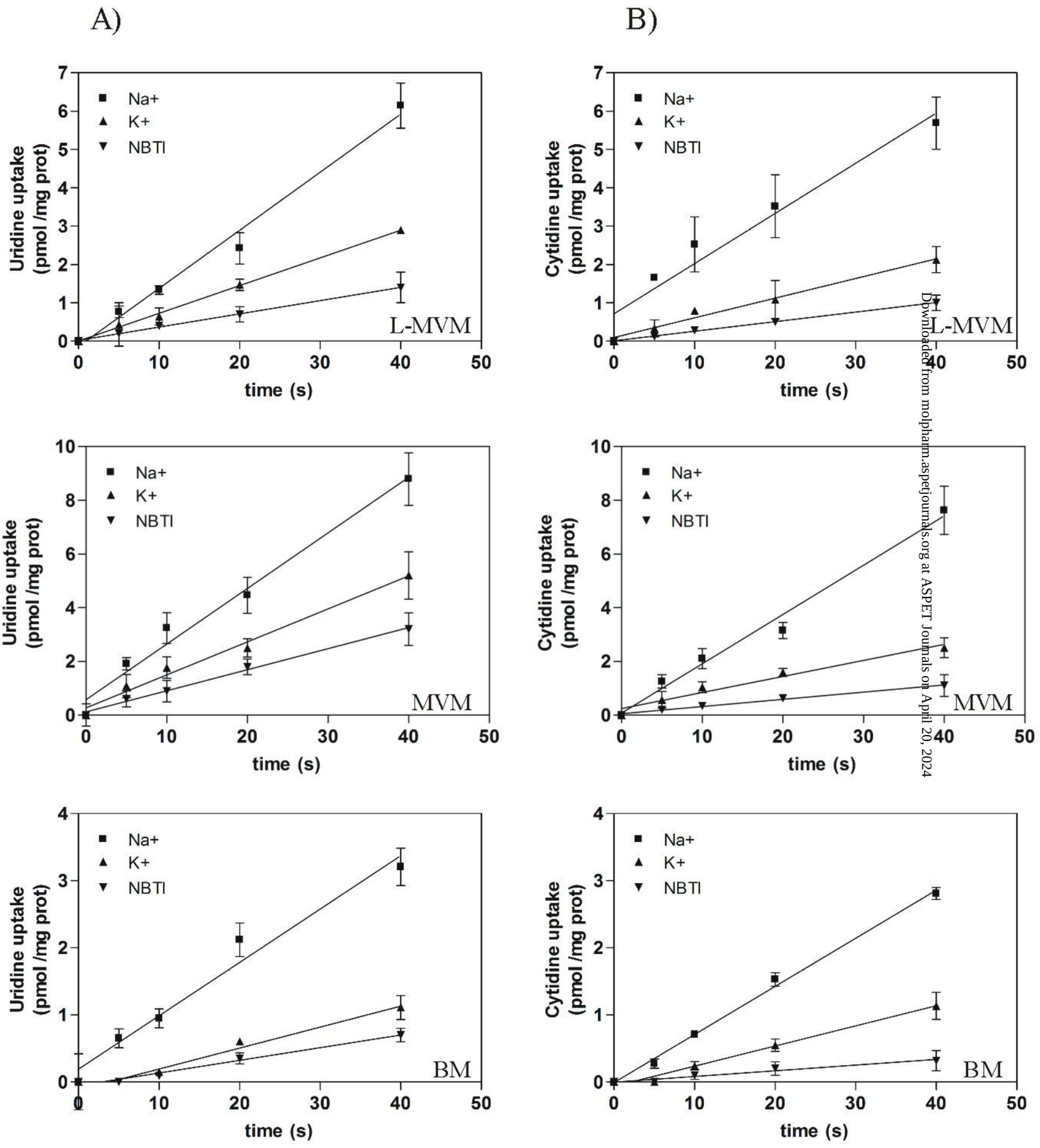

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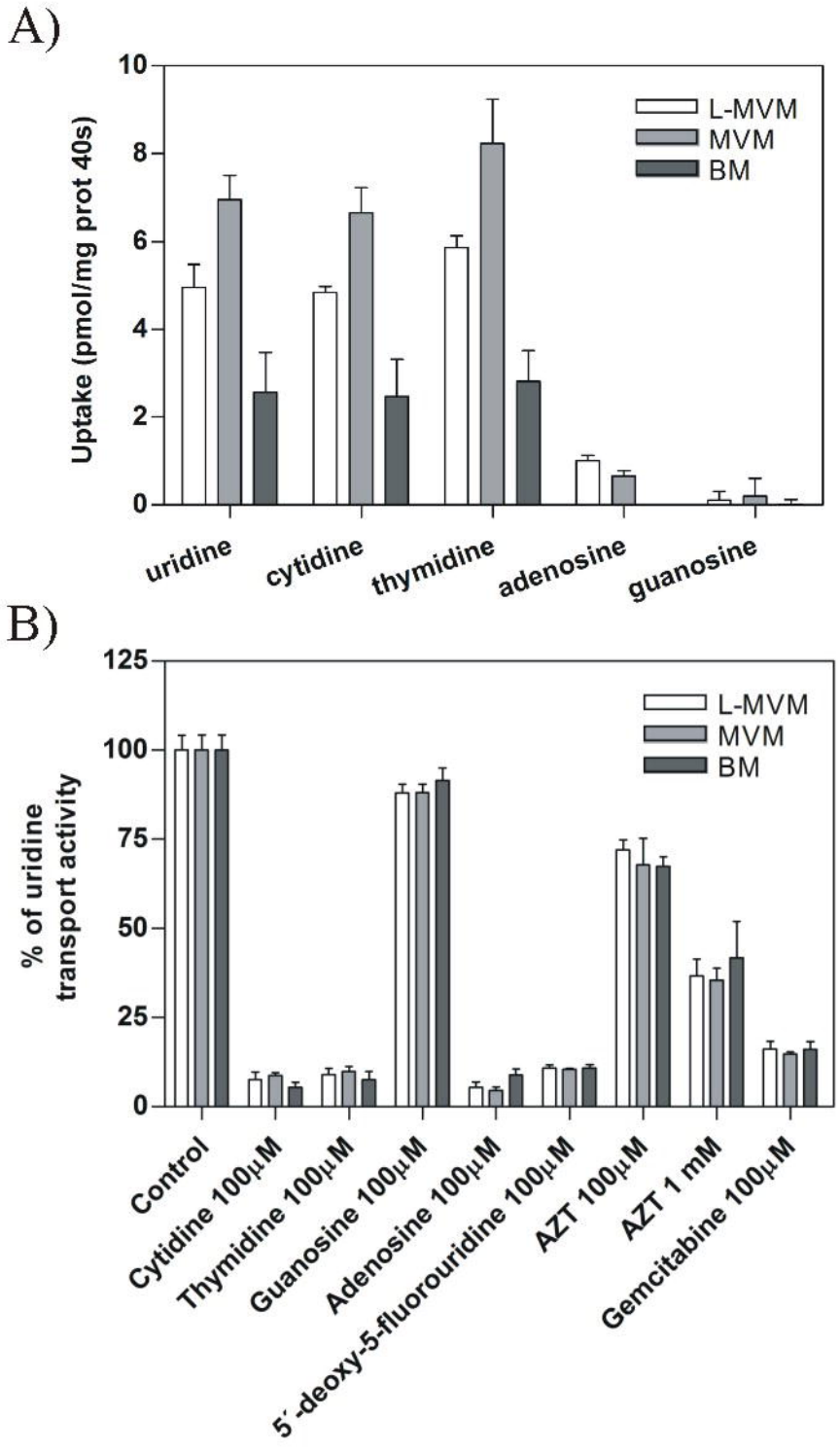
Figure 3


A)
Uptake (pmol/mg prot 40s)
10
8
6
4
2
0
L-MVM
MVM
BM
uridine
cytidine
thymidine
adenosine
guanosine
B)
% of uridine transport activity
125
100
75
50
25
0
L-MVM
MVM
BM
Control
Cytidine 100μM
Thymidine 100μM
Guanosine 100μM
Adenosine 100μM
5´-deoxy-5-fluorouridine 100μM
AZT 100μM
AZT 1 mM
Gemcitabine 100μM

Molecular Pharmacology Fast Forward. Published on August 8, 2011 as DOI: 10.1124/mol.111.071837
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Figure 4

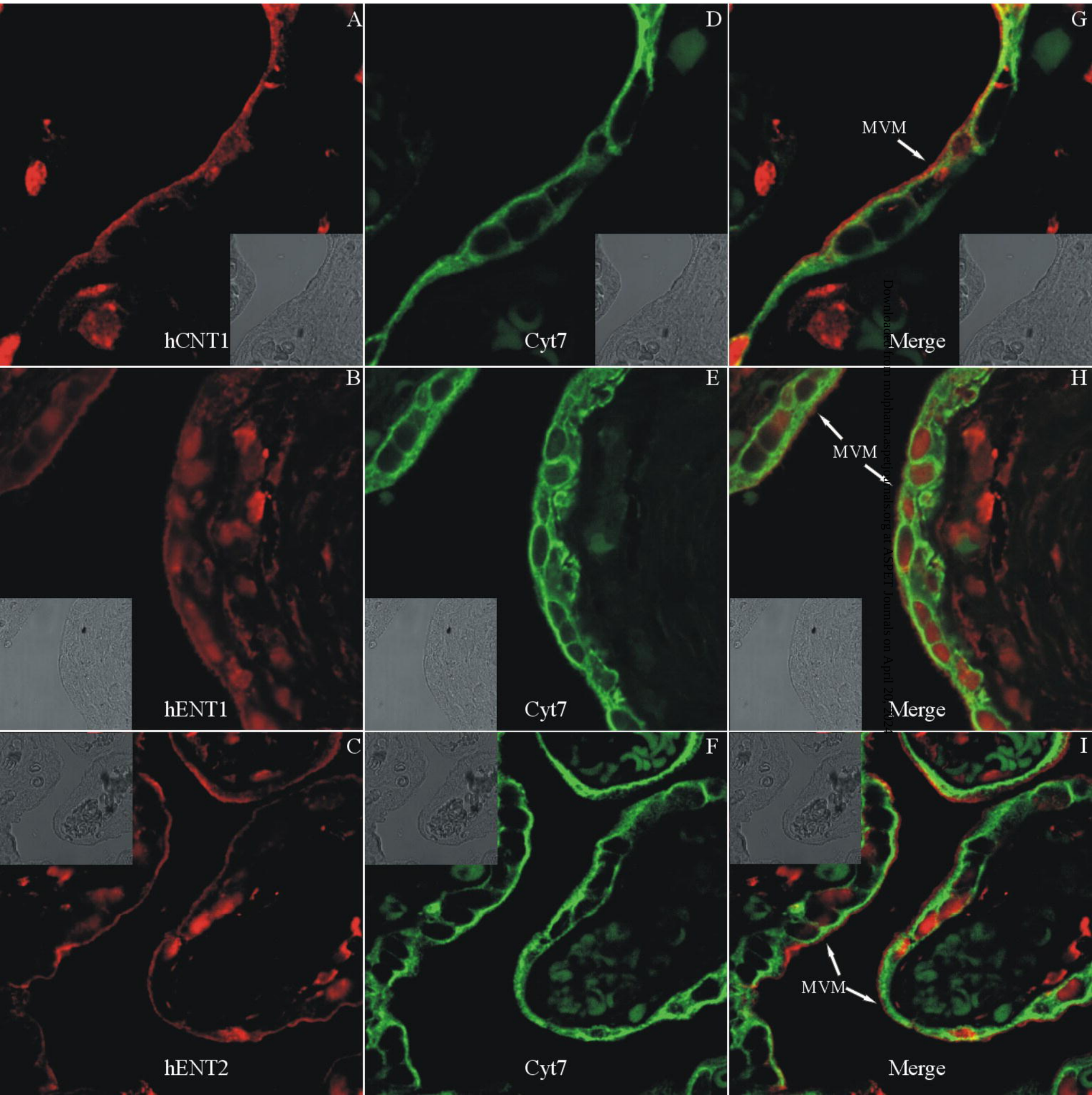

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Figure 5

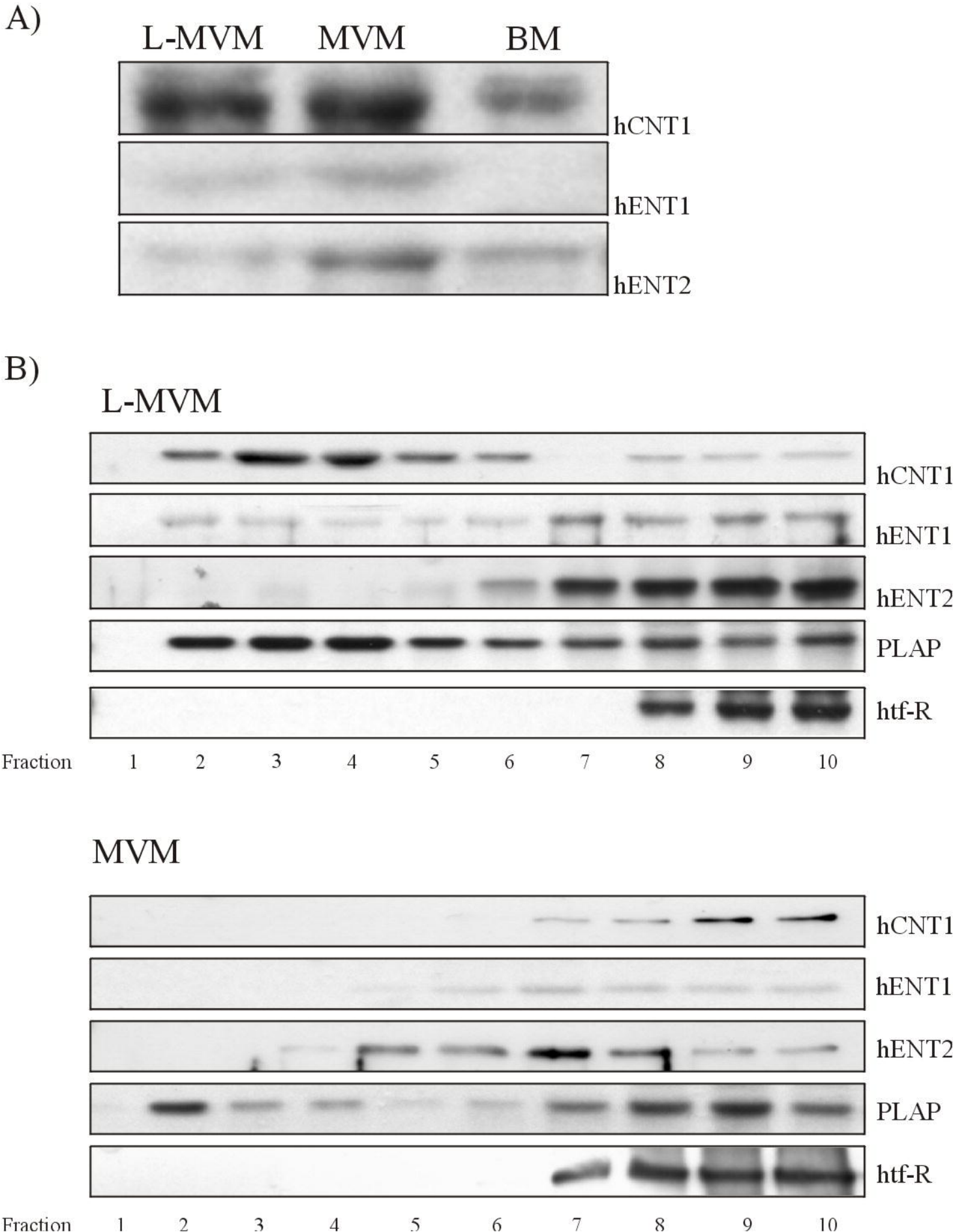
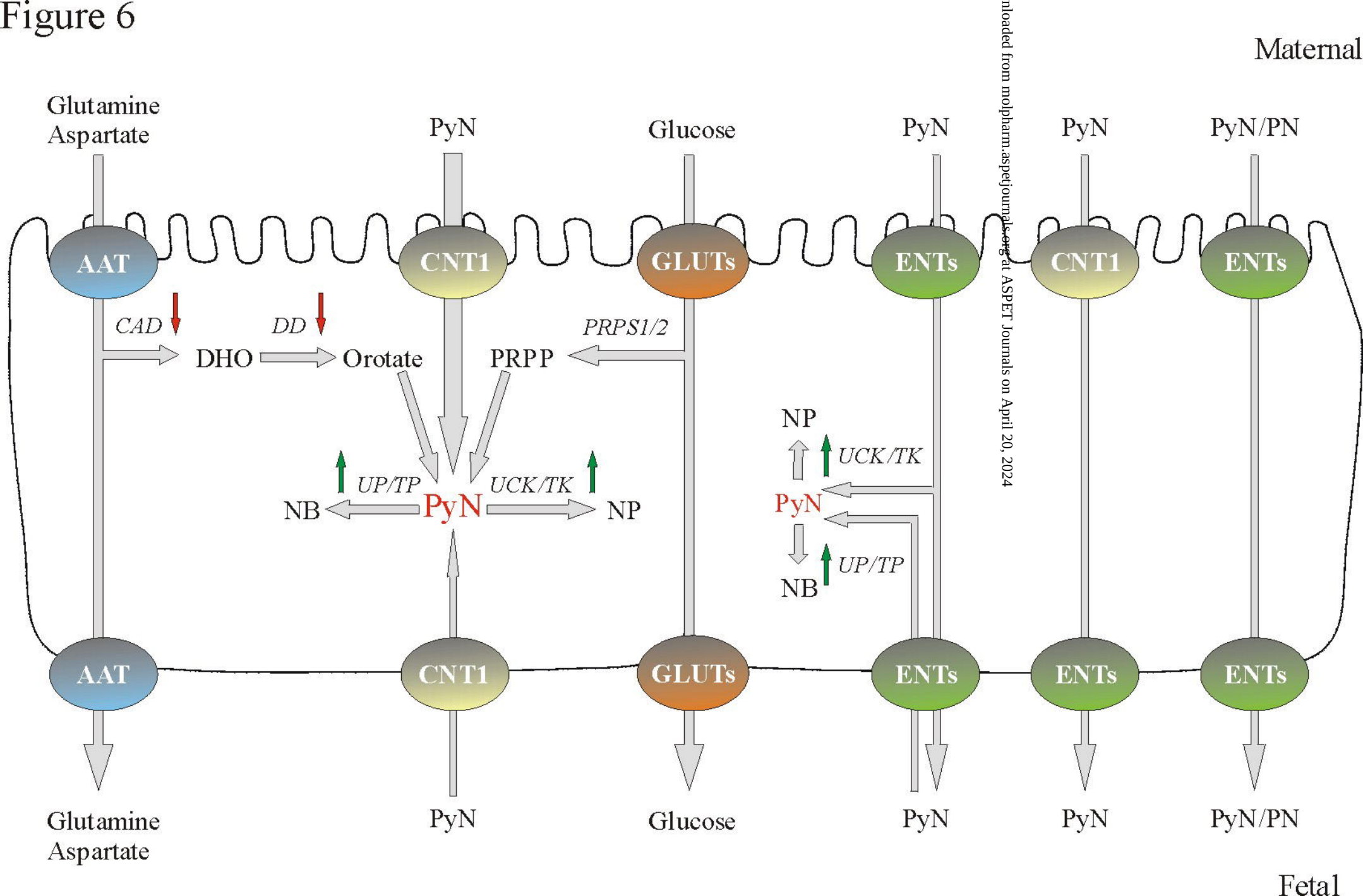


Figure 6



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