Functional Characterization and Haplotype Analysis of Polymorphisms in the Human Equilibrative Nucleoside Transporter, ENT2

Ryan P. Owen, Leah L. Lagpacan, Travis R. Taylor, Melanie de la Cruz, Conrad C. Huang, Michiko Kawamoto, Susan J. Johns, Doug Stryke, Thomas E. Ferrin, and Kathleen M. Giacomini

Department of Biopharmaceutical Sciences (R.P.O., L.L.L., and K.M.G.), and Department of Pharmaceutical Chemistry (T.R.T, M.D., C.C.H., M.K., S.J.J., D.S., and T.E.F.), University of California, San Francisco
Characterization of ENT2 Polymorphisms

Corresponding Author:
Kathleen M. Giacomini
Department of Biopharmaceutical Sciences
University of California
San Francisco, California 94158

Telephone: (415)-476-1936
FAX: (415)-502-4322
email: kmg@itsa.ucsf.edu

Text Pages: 8
Tables: 1
Figures: 4
References: 19

Abstract Words: 250
Introduction Words: 315
Results/Discussion Words: 838

Abbreviations

CLL: Chronic Lymphocytic Leukemia
CNT: Concentrative Nucleoside Transporter
ENT: Equilibrative Nucleoside Transporter
Abstract

The equilibrative nucleoside transporter 2 ENT2 (SLC29A2), is a bidirectional transporter that is involved in the disposition of naturally occurring nucleosides as well as a variety of anticancer and antiviral nucleoside analogs. The goal of the current study was twofold: to evaluate the function of genetic variants in ENT2 in cellular assays, and to determine the haplotype structure of the coding and flanking intronic region of the gene. As part of a large study focused on genetic variation in membrane transporters (Leabman et al., 2003), DNA samples from ethnically diverse populations (100 African-Americans, 100 European-Americans, 30 Asians, 10 Mexicans, and 7 Pacific Islanders) were screened for variants in membrane transporters, including SLC29A2. Fourteen polymorphic sites in SLC29A2 were found, including 11 in the coding region. Five protein-altering variants were identified: three non-synonymous variants, and two deletions. Each of the protein-altering variants was found at a very low frequency, occurring only once in the sample population. The non-synonymous variants and the deletions were constructed via site-directed mutagenesis, and were subsequently characterized in Xenopus laevis oocytes. All variants were able to take up inosine with the exception of ENT2-Δ845-846, which resulted in a frameshift mutation that prematurely truncated the protein. ENT2 showed very infrequent variation compared with most other transporter proteins studied, and it was found that five haplotypes were sufficient to describe the entire sample set. The low overall genetic diversity in SLC29A2 makes it unlikely that variation in the coding region contributes significantly to clinically observed differences in drug response.
Introduction

Synthetic nucleoside analogs are widely used to treat a variety of diseases, including various types of cancer, HIV, Hepatitis C, and other illnesses (Barreiro et al., 2004; Byrd et al., 2004; Li et al., 2004; Pearlman, 2004). Although nucleoside analogs are often the best available therapy, some common problems with nucleoside analog therapies occur, including lack of an initial response, or the development of resistance to therapy. One potential hypothesis for the ineffectiveness of some nucleoside analogs is genetic variation in nucleoside transporters, which function in the uptake of these compounds into cells. Genetic variation could lead to reduced function, or non-functional transporter proteins, which in turn could reduce the amount of drug able to enter the cell and therefore, the intracellular levels of the drug. Genetic variation in both concentrative and equilibrative nucleoside transporter members has been previously examined including CNT1 (Gray et al., 2004), CNT2 (Owen et al., 2005), CNT3 (Badagnani et al., 2005), and ENT1 (Osato et al., 2003).

In this report, we describe the functional characteristics of genetic variants of ENT2 that were identified previously in a large DNA sample set. ENT2 is thought to play a role in nucleoside analog therapy, as it has broad substrate specificity, and is able to transport many of the currently used nucleoside analogs (Baldwin et al., 2004), including the pancreatic cancer drug gemcitabine (Garcia-Manteiga et al., 2003), and fludarabine (Molina-Arcas et al., 2003), used in the treatment of chronic lymphocytic leukemia (CLL). In a recent study, it was reported that expression levels of ENT2, but not ENT1, as measured by a Western Blot, correlated with cytotoxicity of fludarabine in cells
isolated from patients with CLL (Molina-Arcas et al., 2005). This makes studies of genetic variation in ENT2 of particular interest for fludarabine therapy. The aim of this study was to evaluate the functional characteristics of variants of ENT2, and to describe its haplotype structure.
Methods

Genetic Analysis of ENT2. ENT2 variants were identified in the study of Leabman et al. (Leabman et al., 2003) through direct sequencing of its exons and flanking intronic regions in an ethnically diverse population sample of 247 individuals. The nucleotide diversity (π), was calculated as described by Tajima (Tajima, 1989). Synonymous and non-synonymous polymorphisms were defined as described by Hartl and Clark (Hartl and Clark, 1997). Haplotypes were reconstructed from variant positions using PHASE, a Bayesian statistical method (Stephens et al., 2001). Before PHASE analysis, all singletons were removed from the analysis. Five haplotypes were identified through the PHASE analysis. The cladogram describing the ENT2 haplotypes was constructed by hand.

Construction of ENT2-Reference and ENT2-Variant Plasmids. Human ENT2 cDNA was subcloned into the amphibian high-expression vector pOX (Jegla and Salkoff, 1997). ENT2 reference was used as a template to create the three non-synonymous variants, and two deletions of ENT2 identified in the study of Leabman et al (Leabman et al., 2003). Reference and variant sequences were confirmed by complete DNA sequencing at the UCSF Biomolecular Resourse Center.

Functional Screening and Kinetic Studies of Variants in X. laevis Oocytes. Healthy stage V and stage VI X. laevis oocytes were injected with 30-50 ng of capped cRNA transcribed in vitro with T3 RNA polymerase (mCAP™ RNA Capping Kit, Stratagene, La Jolla, CA) from NotI-linearized pOX plasmids containing reference or variant ENT2 (NotI from New England Biolabs, Beverly, MA). Spectrophotometry was used to
determine the concentration of cRNA, and an aliquot of each RNA preparation was run on a 1% agarose gel to ensure that the RNA was not degraded. Injected oocytes were stored in modified Barth’s solution at 18°C (changed 1-2 times daily) for 2-3 days of expression before uptake studies. Seven to nine oocytes were incubated in Na⁺ buffer containing 1 µM ³H substrate. Several different substrates were used including inosine, guanosine, uridine, hypoxanthine, fludarabine, and gemcitabine. All radiolabeled compounds were purchased from Moravek (Brea, CA). The injected oocytes were incubated with ³H substrate (1 µM) for 20 min, ENT2 transport using X. laevis oocytes has been previously reported at 30 min (Yao et al., 2001). In the inhibition study, unlabeled inosine (2 mM) was used to inhibit the uptake of ³H-guanosine (1 µM). For the kinetic studies, 0.25 µM of ³H-inosine was incubated with the unlabeled concentrations of inosine (1, 10, 50, 100, 500, 1000, 2000, 4000 µM), and the uptake was examined for 30 min. The V_max values are reported as pmol/inosine/30 min uptake ± SE. For all the studies with oocytes, uptake was terminated by the removal of buffer containing the radioligand and the oocytes were washed five times in ice-cold choline buffer. Oocytes were then individually lysed by the addition of 10% SDS (100 µL), and the radioactivity associated with each oocyte was determined by scintillation counting. Uptake of all substrates in oocytes expressing each variant was determined in 8-9 oocytes from a single frog. The functional studies were repeated in oocytes from at least one other frog. Data are presented as pmol substrate/oocyte/20 minute uptake, and the error bars indicated are ± SE. Uninjected oocytes incubated with the same reaction mix were used as a control.
Results and Discussion

The variable sites in *SLC29A2* were identified through direct sequencing of all 11 *SLC29A2* exons, and some flanking intronic regions. A summary of all of the total variants identified, as well as the frequency in which they were found in the sample population is shown in Table 1 and can also be found at [http://www.pharmgkb.org](http://www.pharmgkb.org). Of the 14 variable sites, only five resulted in protein-altering variants, and were chosen for further study. All of the protein-altering variants were singletons, or variants that were found on only one chromosome in one individual; three of the five protein-altering variants were found in the African American sample, and the other two were found in the European American sample. ENT2 was unique among the 24 transporters studied by Leabman *et al.* in that it contained two deletion variants in the coding region. In total, 680 polymorphic sites were identified across the 24 transporter genes, but only five of these sites were deletions found in the coding region of the gene, two of which were found in *SLC29A2*. The two deletions likely arose from separate causes because one is near the middle of an exon whereas the other is at the intron-exon boundary. The overall variation in *SLC29A2* was much lower than the average found in the other genes in the studies of Leabman *et al.* (Leabman et al., 2003); the $\pi_T$ of *SLC29A2*, a measure of nucleotide diversity, is $1.64 \times 10^{-4}$, versus the average $\pi_T$ of the genes $5.09 \times 10^{-4}$.

However, the variation in *SLC29A2* was similar to that observed for *SLC29A1* (Osato et al., 2003). The low overall variation in the equilibrative transporter family suggests that these two transporter genes are under high selective pressure, with non-synonymous variation being highly selected against. This contention is also supported by the greater
frequency of synonymous variants in both genes, which would not result in a change in the encoded protein. Although there are no reports of an ENT2 knockout mouse, an ENT1 knockout mouse is viable and fertile (Choi et al., 2004). Consistent with its low variability, ENT2 has few haplotypes (in the coding and flanking intronic region), which are shown in the cladogram in Figure 1. The *1 and *2 haplotypes account for nearly 95% of the overall haplotypes found, with *1 comprising about 75% of the population sampled. This haplotype profile is similar to that observed for ENT1, but showed considerably lower variability than the haplotype profiles of CNT family members (Gray et al., 2004), (Owen et al., 2005), (Badagnani et al., 2005).

Functional studies in oocytes revealed that ENT2-Δ845-846 was not able to take up inosine (or guanosine). In contrast, the other variants (as well as ENT2-reference) were able to transport both substrates (Figures 2a and 2b). ENT2-Δ845-846 was unable to transport inosine, because the deletion of two base pairs produced a change in the reading frame, which results in a severe truncation of the protein, and a subsequent loss of function. The variants ENT2-D5Y and ENT2-Δ551-556 showed reduced inosine uptake when compared to ENT2-reference, with ENT2-D5Y reaching statistical significance: p=0.048 for ENT2-D5Y and p=0.061 for ENT-Δ551-556 (Figure 2a). The loss of six base pairs in ENT2-Δ551-556 results in a two amino acid deletion, and a non-synonymous change of a third residue.

The uptake of a diverse array of substrates by ENT2 and its variants was examined (Figure 3). Included in the analysis was a model purine (inosine), a model pyrimidine...
(uridine), the nucleobase hypoxanthine, as well as the nucleoside analog drugs gemcitabine and fludarabine. Transport profiles were similar regardless of the substrate, with ENT2-D5Y exhibiting reduced function for all tested substrates, and ENT2-Δ845-846 not transporting any of the substrate panel. In order to gain insight into the mechanism of the reduced function of ENT2-D5Y, we performed kinetic studies with ENT2-reference and ENT2-D5Y with inosine and fludarabine. Both compounds showed a reduced $V_{\text{max}}$ for ENT2-D5Y relative to that of ENT2-reference. Representative curves of the inosine kinetics with ENT2-reference, ENT2-D5Y, and uninjected oocytes are shown in Figure 4. The observed difference in $V_{\text{max}}$ (958 ± 53.6 pmol inosine/oocyte/30 min uptake vs. 706 ± 68.1 pmol inosine/oocyte/30 min uptake for ENT2-reference and ENT2-D5Y respectively) is statistically significant (p=0.03), while the respective $K_m$’s were not statistically significant (p=0.45). These data support that the mechanism of reduction in activity of ENT2-D5Y is due to a reduced $V_{\text{max}}$, possibly reflecting a reduced turnover rate constant or a reduction in the number of functional transporters expressed on the plasma membrane.

In summary, the low genetic and functional variation observed in ENT2 suggests a critical physiological role, similar to its homolog ENT1. Variants with altered function were observed in ENT2; however, because of their low frequency, these variants are unlikely to be a major source of variability in drug response. Our data do not explain previous studies in which enhanced ENT2 expression has been associated with response to anticancer drugs (Molina-Arcas et al., 2005). It is possible that polymorphisms in noncoding regions of ENT2 may explain variation in the expression levels of this gene.
Acknowledgements

We would like to thank Ilaria Badagnani and Jennifer Gray for helpful discussions.
References


variants in the human equilibrative nucleoside transporter, ENT1. 

Pharmacogenetics 13:297-301.


Footnotes

This work was funded by the National Institute of Health grants GM61390 and GM42230
Figure Legends

**Figure 1: Cladogram of ENT2.** PHASE analysis of the ENT2 haplotypes generated only five distinct haplotypes. *1 accounted for the largest fraction of the population sample, and is considered to be reference ENT2. The *2 and *3 haplotypes have a base pair substitution in the flanking intronic regions. The *4 and *5 haplotype each encode a synonymous change, and are found at much lower frequency. The circumference of the circle is proportional to the frequency with which the haplotype was found within the sample population. The *1 and *2 haplotypes were found in every ethnic group, but *3, *4, and *5 were found in the African American sample only. None of the protein-altering variants that we examined are included in the haplotype structure because all singletons were removed prior to haplotype analysis.

**Figure 2a: Uptake of inosine in oocytes expressing the reference ENT2 and five protein altering variants.** Oocytes injected with cRNA encoding ENT2-reference, all five protein-altering variants of ENT2, and uninjected oocytes were incubated with \(^{3}\text{H}\)-inosine (1 µM). ENT2-Δ845-846 was not able to transport inosine.

**Figure 2b: Transport of guanosine by ENT2 and its variants is inhibited by inosine.** The uptake of \(^{3}\text{H}\)-guanosine (1 µM) by ENT2-reference and its variants was inhibited by the addition of inosine (2 mM). Open bars represent the uptake of
radiolabeled guanosine alone, and dark bars indicate the uptake of radiolabeled guanosine inhibited by inosine.

**Figure 3: Uptake of a panel of substrates by ENT2-reference and its coding region variants.** ENT2-reference, and its variants are able to transport a variety of different compounds. The bars are coded according to the legend to the right of the figure. ENT2-Δ845-846 is unable to transport any of the substrates, whereas all the other variants can, ENT2-D5Y appears to have globally reduced function, and ENT2-Δ551-556 may have a reduced affinity for inosine relative to the other substrates.

**Figure 4: Inosine kinetics by ENT2-reference and ENT2-D5Y.** Oocytes injected with either ENT2-reference or ENT2-D5Y were incubated with 0.25 µM ³H-inosine with eight different concentrations of unlabeled inosine (1, 10, 50, 100, 500, 1000, 2000, and 4000 µM) for 30 min, and the counts associated with each concentration were plotted. Uninjected oocytes were used as a control to show the low background transport of the oocytes. Error bars represent standard error for each condition. The V_max of ENT2-reference is significantly higher than the V_max of ENT2-D5Y (p=0.03).
Table 1: Genetic variants in ENT2 identified in DNA samples from 247 ethnically diverse subjects. The exon in which the change occurred, as well as the position of the nucleotide in the exon or flanking intronic region is also shown. The amino acid residue affected by the nucleotide change is indicated where appropriate, and the nature of the change is described. The frequency at which each variant occurred in the sample population is also indicated; a frequency of .002 indicates that the variant was found on one chromosome in one individual.
Figure 3

Graph showing pmol nucleoside/oocyte/20 min uptake for different treatments:
- Inosine
- Uridine
- Hypoxanthine
- Fludarabine
- Gemcitabine

Treatments includes:
- ENT2-reference
- ENT2-D5Y
- ENT2-N68K
- ENT2-P94L
- ENT2-Δ551-556
- ENT2-Δ845-846
- uninjected