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Stereoselective Binding of Chiral Ligands to Single Nucleotide Polymorphs (SNPs) of the Human Organic Cation Transporter-1 Determined Using Cellular Membrane Affinity Chromatography

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Abstract

Membranes from stably transfected cell lines that expresses two point mutations of the human organic cation 1 transporter (hOCT1), R488M and G465R, have been immobilized on the immobilized artificial membrane (IAM) liquid chromatographic stationary phase to form the Cellular Membrane Affinity Chromatography (CMAC) (hOCT1_{G465R}) and CMAC(hOCT1_{R488M}). Columns were created using both stationary phases and frontal displacement chromatography experiments were conducted using [³H]-methyl phenyl pyridinium, [³H]-MPP⁺, as the marker ligand and various displacers, including the single enantiomers of verapamil, fenoterol and isoproterenol. The chromatographic data obtained was used to refine a previously developed pharmacophore for the hOCT1 transporter.

Introduction

Single-nucleotide polymorphisms (SNPs) are single nucleotide variations in a DNA sequence. Recent studies have associated SNP variations in receptor proteins with susceptibility to diseases such as Alzheimer's disease [1] and schizophrenia [2], and a relationship between brain function and genomic SNP factors has been demonstrated using functional magnetic resonance imaging and SNP arrays [3]. SNP variants can also affect the activities of enzymes and drug transporters such as the human organic cation transporter (hOCT) SLC22 family [4–7]. The SLC22 family is composed of transporters that mediate the bidirectional transport of small organic cations (50–350 amu) such as tetraethylammonium (TEA) and 1-methyl-4-phenylpyridinium (MPP⁺) and includes the hOCT1, hOCT2, hOCT3 and hOCTN1 subtypes [4,5]. These proteins are found in the liver, kidney and intestines and play a major role in the metabolism and excretion of clinically important therapeutics, endogenous compounds and toxic substances [4–6].

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The effect of SNP variations on the transport activity of the hOCT subtypes has been investigated by following the transport of [^{14}C]-TEA and [^3H]-MPP $^+$ in oocytes [4,5], MDCK cells [5] or HEK293 cells [7] transfected with the SNP variants. The data indicate that the effect on hOCT transport is dependent upon the type and position of the mutation. For example, Kang, et al. [4] expressed four hOCT1 and three hOCT2 SNP variants in oocytes and two of the four hOCT1 and all of the hOCT2 variants showed decreased [^3H]-MPP $^+$ uptake with 2- to 5-fold increase in K_m values and 10- to 20-fold decrease in $V_{\max}$ values. In addition, Shu, et al. [5] expressed 15 hOCT1 SNP variants and determined the effect on transport activity using [^3H]-MPP $^+$. The results indicated that the effect of a SNP variation on transport activity was a function of the type and location of the amino acid substitution [5].

It has been demonstrated that hOCT transport can be competitively inhibited by a diverse group of commonly administered drugs including verapamil, quinidine, quinine and disopyramide [8]. This inhibition is also stereoselective, which has been demonstrated by functional studies [8,9] and chromatographic studies [9–11]. In the latter studies membrane fragments from a stably transfected MDCK cell line expressing hOCT1 were immobilized on an immobilized artificial membrane stationary phase to create a cellular membrane affinity column, CMAC (hOCT1) [9–11]. This technique and the experimental procedures associated with the creation and use of CMAC columns have recently been reviewed [12].

In the current study, membrane fragments obtained from stably transfected MDCK cell lines expressing two hOCT1 SNP variants, hOCT1-R488M and hOCT1-G465R [5], were used to create two CMAC columns, CMAC(hOCT1_{R488M}) and CMAC(hOCT1_{G465R}). These proteins were chosen as the R488M substitution did not affect transport function while the G465R mutant exhibited decreased function [5]. The results of this study indicate that the CMAC (hOCT1_{G465R}) retained the ability to stereoselectively bind a set of chiral ligands and that this observed selectivity was the same as the wild-type hOCT1, CMAC(hOCT1). The CMAC (hOCT1_{R488M}) also retained the ability to stereoselectively bind the chiral ligands, but with a selectivity that was opposite to that observed on the CMAC(hOCT1) and CMAC (hOCT1_{G465R}). The data was used to refine the previously reported pharmacophore model of the stereoselective binding to the hOCT1 [9].

Materials and Methods

Materials

(R)-Verapamil, (S)-verapamil, (R)-isoproterenol, (S)-isoproterenol, quinine, quinidine, N-methyl-4-phenyl pyridinium acetate (MPP $^+$), the inorganic salts, Tris-HCl buffer, CHAPS and PBS were purchased from Sigma Chemical Company (St. Louis, MO, USA). [^3H]-MPP $^+$ was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO, USA). Immobilized artificial membrane stationary phase (IAM-PC, 12 micron particle size, 300 Å pore size) was purchased from Regis Technologies Inc. (Morton Grove, IL, USA). HR 5/2 glass columns were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Cell lines

MDCK cells stably transfected with cDNA expressing the hOCT1-G465R and hOCT1-R488M SNPs have been previously described [5] and were provided by K. Giacomini (Department of Biopharmaceutical Sciences, University of California San Francisco, San Francisco, CA, USA).

Preparation of CMAC(hOCT1_{R488M}) and CMAC(hOCT1_{G465R}) Columns

The CMAC(hOCT1_{R488M}) and CMAC(hOCT1_{G465R}) columns were prepared following the previously reported method for the creation of the CMAC(hOCT1) column [10,12]. In brief,

solubilized membranes were prepared from hOCT1-R488M or hOCT1-G465R hOCT1 cells (100×10^6 cells). The solution containing the solubilized membranes was added to 160 mg of the IAM stationary phase, the resulting mixture was rotated at room temperature for 3 h at 150 rpm using an orbit shaker and then dialyzed against Tris HCl [50 mM, pH 7.4] containing 150 mM NaCl, 1 mM EDTA, 1 mM benzamidine for 2 days. The resulting mixture was centrifuged for 3 min at 4 °C at $700 \times g$ and the supernatant was discarded. The stationary phase pellet was washed with Tris-HCL [10 mM, pH 7.4] containing 1 mM CaCl_2 , 0.5 mM MgCl_2 and centrifuged. This process was repeated until the supernatant was clear. The stationary phase (160 mg) was packed into a HR 5/2 glass column to yield a 150 mm $\times$ 5 mm (ID) chromatographic bed.

Chromatographic Studies

The chromatographic studies were carried out as previously described [10,12]. In brief, the CMAC(hOCT1_{R488M}) and CMAC(hOCT1_{G465R}) columns were connected to a LC-10AD isocratic HPLC pump (Shimadzu, Columbia, MD, USA). The mobile phase consisted of Tris-HCL [10 mM, pH 7.4] containing 1 mM CaCl_2 , 0.5 mM MgCl_2 delivered at 0.2 ml/min at room temperature. Detection of the marker ligand [^3H]-MPP⁺ (20 pM) was accomplished using an on-line scintillation detector (IN/US system, β -ram Model 3, Tampa, FL, USA) with a dwell time of 2 s using Laura lite 3. A 50-ml Superloop (Amersham Pharmacia Biotech) was used to apply the marker ligand and the displacer ligands to the CMAC columns in the following concentrations:

CMAC(hOCT1_{R488M})—(R)- and (S)-verapamil: 1 μM , 2.5 μM , 5.0 μM , 7.5 μM , 10.0 μM , 20.0 μM ; (R)-isoproterenol: 25 μM , 50 μM , 100 μM , 150 μM , 200 μM , 300 μM ; (S)-isoproterenol: 500 μM , 700 μM , 800 μM , 1 mM; (S,S)-fenoterol: 5.0 μM , 10.0 μM , 20.0 μM ; (R,R)-fenoterol: 5.0 μM , 10.0 μM , 15.0 μM , 20.0 μM , 25.0 μM ; 40 μM , (S,R)-fenoterol: 5.0 μM , 10.0 μM , 20.0 μM ; (R,S)-fenoterol: 1.5 μM , 2.5 μM , 5.5 μM , 10.0 μM , 20.0 μM , 40 μM ; quinine: 1 μM , 4.0 μM , 10 μM , 20.0 μM , 40.0 μM ; quinidine: 25 μM , 50 μM , 100 μM , 150 μM , 300 μM .

CMAC(hOCT1_{G465R})—(R)- verapamil: 0.05 μM , 0.1 μM , 0.5 μM , 0.75 μM , 1.0 μM ; (S)-verapamil: 1.0 μM , 2.5 μM , 5.0 μM , 7.5 μM , 15 μM ; (S,S)-fenoterol: 5.0 μM , 10.0 μM , 20.0 μM ; (R,R)-fenoterol: 5.0 μM , 10.0 μM , 15.0 μM , 20.0 μM , 25.0 μM ; 40 μM , (S,R)-fenoterol: 5.0 μM , 10.0 μM , 20.0 μM ; (R,S)-fenoterol: 1.5 μM , 2.5 μM , 5.5 μM , 10.0 μM , 20.0 μM , 40 μM .

Data Analysis

The dissociation constants, K_d , for the displacer ligands were calculated using a previously described approach [12]. The data was analyzed by non-linear regression with a sigmoidal response curve using Prism 4 software (Graph Pad Software, Inc., San Diego, CA, USA) running on a personal computer.

Molecular Modeling

Pharmacophore modeling was carried out using Catalyst version 4.11 (Accelrys Software Inc.) as previously described [9]. All the modeling were carried out on a SGI workstation (Octane) running IRIX 6.5.28m operating system and having a 400 MHz processor with CPU MIPS R12K. Catalyst module, HypoGen was used to construct the models. The models presented in this study were created by modifying the basic pharmacophore (see Figure 2) consisting of a hydrophobic, a positive ion and two hydrogen-bond acceptor sites (HBA1 and HBA2). Two new pharmacophore models were manually created from this basic model one in which the HBA1 site had been removed and the HBA2 site retained, model (HBA1(−)), and the other in

which the HBA2 site was removed and the HBA1 site retained, model (HBA2(-)). The new pharmacophore models [HBA1(-) and HBA2(-)] were created and the analyses were carried out using Accelrys Discovery Studio (version 2.5) software. Discovery Studio (Accelrys Software Inc) was installed on a Dell Precision PWS380 with Intel Pentium CPU of 3GHz and 1 GB of RAM running Windows XP.

Results and Discussion

The preparation and use of CMAC columns have been recently reviewed and the experimental protocols presented and discussed [12]. In brief, in frontal chromatography a marker ligand that is specific for the immobilized target is placed in the mobile phase and passed through the column and elution profiles containing front and plateau regions are then obtained. The frontal region is the relatively flat initial portion of the chromatographic trace that reflects the binding of the marker to the target up to saturation, which is represented by a vertical breakthrough and attainment of a plateau region. In a competitive displacement study, increasing concentrations of a competing ligand are added to the mobile phase and the effects on the retention volume of the marker, measured at the mid-point of the breakthrough curves, are determined and the affinity of the displacer (K_d value) for the site(s) that the marker binds can be calculated from the relationship between displacer concentration and changes in the retention volume as can the number of active binding sites, B_{max} .

In this study, the immobilized targets were hOCT1-R488M and hOCT1-G465R and the specific marker ligand was [^3H]-MPP $^+$. When [^3H]-MPP $^+$ was chromatographed on the CMAC (hOCT1_{R488M}) and CMAC(hOCT1_{G465R}) columns, chromatographic traces containing front and plateau regions were obtained indicating that the marker specifically bound to the immobilized transporters (data not shown). The affinity of MPP $^+$ to the immobilized proteins was determined using displacement chromatographic studies with MPP $^+$ as the displacer. The calculated K_d values were 0.17 μM and 0.38 μM for the CMAC (hOCT1_{R488M}) and CMAC (hOCT1_{G465R}) columns, respectively, indicating that the immobilized transporters retained their abilities to specifically bind MPP $^+$. The calculated B_{max} values were 344 pM {CMAC (hOCT1_{R488M})} and 485 pM {CMAC(hOCT1_{R488M})}, which were similar to the 512 pM active binding sites found on the initial CMAC(hOCT1) column.

Since membrane fragments, and not purified proteins, are used to create CMAC columns, both specific and non-specific interactions can occur between the marker ligand and the immobilized membrane. In the initial studies with the CMAC(hOCT1) column, the membranes used to create the stationary phase were obtained from a stably transfected MDCK cell line [10]. A control column was also prepared using membrane fragments obtained for native MDCK cells, CMAC(MDCK). The data from the initial study indicated that the marker ligand, [^3H]-MPP $^+$, bound to both non-specific and specific sites, other than the hOCT1, on the CMAC (MDCK) column. The results also indicated that, even in the presence of these background interactions, the affinities of specific hOCT1 ligands for the immobilized transporter could be determined and were directly comparable to data obtained from cellular uptake studies, $r^2 = 0.9987$ ($p < 0.001$) [10]. Since the membranes used in this study were obtained from MDCK cells stably expressing hOCT1-R488M or hOCT1-G465R [5], it was assumed that the same background interactions would be present in the CMAC(hOCT1_{R488M}) and CMAC (hOCT1_{R488M}) columns.

The initial studies with the CMAC (hOCT1) column also demonstrated that the binding of some chiral inhibitors to the immobilized transporter was stereoselective [9–11] and that this stereoselectivity was reflected to enantioselective functional inhibition of hOCT1 transport [11]. Since the physicochemical properties of enantiomeric compounds are essentially equivalent and since the background interactions with the MDCK membranes should be

consistent between the hOCT1 containing CMAC columns, the enantioselectivities of the CMAC(hOCT1), CMAC(hOCT1_{R488M}) and CMAC(hOCT1_{G465R}) columns were used to characterize the effects of the SNP variations on the binding of ligands to the proteins. Thus, the K_d values of (R)- and (S)-verapamil, (R)- and (S)-isoproterenol, (R,R)- and (S,S)-fenoterol and (R,S)- and (S,R)-fenoterol were determined on the CMAC (hOCT1_{R488M}) and CMAC (hOCT1_{G465R}) columns and compared to previously reported data obtained on the CMAC (hOCT1) [9], Table 1. Although the physicochemical properties of diastereomers are not assumed to be equivalent, the K_d values of the diastereomers quinine and quinidine were also determined on the CMAC(hOCT1_{R488M}) column, Table 1.

Frontal displacement curves of the [³H]-MPP⁺ marker were observed for all of the compounds chromatographed on the CMAC(hOCT1_{G465R}). Representative chromatographic traces produced by 1 μ M (R)- and (S)-verapamil are presented in Figure 1A. The K_d values calculated for (R)- and (S)-verapamil, (R,R)- and (S,S)-fenoterol and (R,S)- and (S,R)-fenoterol calculated from the displacement studies on the CMAC(hOCT1_{G465R}) column did not significantly differ from the affinities determined using the CMAC(hOCT1), Table 1. The enantioselectivities and diastereoselectivities of the binding interactions were determined as the relative affinities, e.g. K_d (S)-verapamil/ K_d (R)-verapamil, and expressed as the stereoselectivity factor α . The observed α values for the compounds used in this study did not significantly differ from the results obtained on the CMAC(hOCT1), Table 2. The data indicates that while the transport activity of the hOCT1-G465R mutant was reduced by the alteration, the binding of the inhibitors used in this study was not affected.

Frontal displacement curves of the [³H]-MPP⁺ marker were observed for all of the compounds chromatographed on the CMAC(hOCT1_{R488M}) column. Representative chromatographic traces produced by 1 μ M (R)- and (S)-verapamil are presented in Figure 1B. The K_d values calculated from the displacement studies on the CMAC (hOCT1_{R488M}) column for (S)-verapamil, (R,R)-fenoterol, (S,R)-fenoterol and quinine were not significantly different from the same data obtained on the CMAC(hOCT1) column, Table 1. However the binding affinities of (R)-verapamil, (S,S)-fenoterol and quinidine were decreased while the affinities of (R,S)-fenoterol, (S)-isoproterenol and (R)-isoproterenol were increased, although the effect on the K_d of (S)-isoproterenol was greater than that on (R)-isoproterenol, Table 1. The observed α values for all of the compounds were affected, although the changes in α were <2-fold for (R,S)-isoproterenol and (S,R)/(R,R)-fenoterol, Table 2. The results of this study indicate that the binding of compounds to the hOCT1-R488M can be significantly altered relative to the wild-type hOCT1. Since hOCT1-R488M is an active transporter and the compounds used in this study represent some clinically relevant compounds, the results suggest that the CMAC approach can be used to screen the effect of SNP variations on potential drug-drug interactions.

CMAC(hOCT1) determined stereoselectivity has been previously used to construct a pharmacophore model that describes and predicts stereoselective binding to the hOCT1 [11]. The resulting model was composed of a positive ion interaction site, a hydrophobic interaction site and two hydrogen-bond acceptor sites, HBA1 and HBA2, Fig. 2. In this model, (R)-verapamil mapped to all four of the postulated sites, while (S)-verapamil mapped to only three sites, the positive ion, hydrophobic and HBA2 sites. The observed enantioselectivity was attributed to the mapping of the nitrile moiety on the chiral carbon of (R)-verapamil to the HBA1 site, while the corresponding interaction was not achieved by the (S)-enantiomer [9, 11,13].

In the hOCT1-G465R variant, the replacement of a neutral glycine residue by a basic arginine moiety had no effect on the enantiomeric selectivity of verapamil on the CMAC (hOCT1_{G465R}) column. Since the G465 residue is believed to be located on the intracellular portion of the protein [5], the data suggest that the G465R mutation is located far enough from

the inhibitor binding site to have any effect on the binding interactions and so this residue interaction cannot be part of the previously proposed pharmacophore.

Conversely, the substitution of a basic arginine residue for a neutral methionine residue in hOCT1-R488M had a significant effect on the enantioselective binding of (R)- and (S)-verapamil to the CMAC(hOCT1_{R488M}) column, as the removal of the potential hydrogen-bond accepting site reduced the affinity of (R)-verapamil by almost 40-fold but had no significant effect on the binding of the (S)-enantiomer, Table 1. These results suggested that the R488M mutation may affect the HBA1 or HBA2 site in the proposed pharmacophore. This possibility was explored through the creation of two new pharmacophore models, one in which the HBA1 site had been removed and the HBA2 site retained, model (HBA1(−) model), and the other in which the HBA2 site was removed and the HBA1 site retained, model(HBA2(−) model). The enantiomers of verapamil were docked in both of the revised pharmacophores, Fig 3 and Fig 4, respectively, and both of the enantiomers mapped to the three sites in the respective models, a positive ion interaction site, a hydrophobic interaction site and a hydrogen-bond acceptor site. The calculated activities of the (R)- and (S)-verapamil complexes with model (HBA1(−)) were similar at 2.73 and 2.91 μ M respectively, indicating that the model did not reflect enantioselectivity in the binding interactions. When (R)-verapamil was docked into the model (HBA2(−)) the estimated activity was 1.81 μ M while the calculated activity of the (S)-verapamil complex was 4.50 μ M. The results obtained with the HBA2(−) model pharmacophore are consistent with chromatographic data and suggest that the site identified as HBA2 in the proposed hOCT1 pharmacophore is associated with R488 in the hOCT1 protein. The data are also consistent with the previously postulated conformation-driven chiral recognition mechanism describing enantioselective ligand-hOCT1 interactions observed on the CMAC(hOCT1) column [13]. In this mechanism, chiral recognition is a multi-step process involving an initial tethering of the ligand to the hOCT1 protein, most probably at the positive ion interaction site, followed by conformational adjustments which produce the optimum interactions with a combination of the hydrophobic, HBA1 and HBA2 sites. The observed enantioselectivities are the result of the relative conformational energies required to optimize the binding interactions.

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Figure 1.

A. The effect of the addition of 1 μM (R)-verapamil (A) and 1.0 μM (S)-verapamil (B) to the mobile phase on the chromatographic retention of 20 pM $[^3\text{H}]\text{-MPP}^+$ on the CMAC (hOCT1_{G465R}) column. **B.** The effect of the addition of 10 μM (R)-verapamil (A) and 10.0 μM (S)-verapamil (B) to the mobile phase on the chromatographic retention of 20 pM $[^3\text{H}]\text{-MPP}^+$ on the CMAC (hOCT1_{R488M}) column.

Figure 2.

The pharmacophore model reprinted from Ref [9] in which the red sphere represents a positive ion interaction site, the blue sphere represents a hydrophobic interaction site and the green spheres represent two hydrogen-bond acceptor sites, HBA1 and HBA2.

A.

B.

Figure 3.

The fit of verapamil enantiomers in the proposed pharmacophore without HBA1 (HBA1(-) model), where: A = the mapping of (R)-verapamil; B = the mapping of (S)-verapamil. Non-polar hydrogen atoms have been omitted for clarity.

A.

B.

Figure 4. The fit of verapamil enantiomers in the proposed pharmacophore without HBA2 (HBA2(−) model), where: A = the mapping of (R)-verapamil; B = the mapping of (S)-verapamil. Non-polar hydrogen atoms have been omitted for clarity.

Table 1

The binding affinities, K_d values, of the compounds used in this study for the immobilized hOCT1 and hOCT1-G465R and hOCT1-R448M SNP variants determined on the CMAC(hOCT1), CMAC(hOCT1_{G465R}) and CMAC(hOCT1_{R448M}) columns. The data was obtained using frontal displacement chromatography with [³H]-MPP⁺ as the marker ligand; see text for experimental details.

	CMAC(hOCT1) ^a K_d [μ M]	CMAC(hOCT1 _{G465R}) K_d [μ M]	CMAC(hOCT1 _{R448M}) K_d [μ M]
(S)-Verapamil	3.46	2.84	8.12
(R)-Verapamil	0.04	0.05	1.82
(R,R)- Fenoterol	12.6	13.4	8.60
(S,S)-Fenoterol	3.73	6.35	32.8
(S,R)-Fenoterol	6.18	1.67	7.17
(R,S)-Fenoterol	13.2	10.4	0.96
(S)-Isoproterenol	180	ND	50.8
(R)-Isoproterenol	120	ND	59.9
Quinine	10.2	ND	19.9
Quinidine	6.33	ND	112

^a Previously reported in reference [9]

^b ND = Not Determined

Table 2

The stereoselective binding to the immobilized hOCT1 transporter and hOCT1-G465R and hOCT1-R448M SNP variants determined using cellular membrane chromatography on the CMAC(hOCT1), CMAC(hOCT1_{G465R}) and CMAC(hOCT1_{R448M}) columns. Where the stereoselectivity (α) for the enantiomeric pairs is defined as $K_d(S)/K_d(R)$ and for the diastereomeric pairs, the ratios are as presented in the table.

	CMAC(hOCT1) ^a α	CMAC(hOCT1 _{G465R}) α	CMAC(hOCT1 _{R448M}) α
(R,S)-Verapamil	86.5	56.8	4.46
(R,R;S,S)-Fenoterol	0.3	0.47	3.81
(R,S;S,R)-Fenoterol	0.49	0.16	7.47
(R,S)-Isoproterenol	1.50	ND ^b	0.85
Quinine/quinidine	1.60	ND ^b	0.17
(R,S)/(R,R) Fenoterol	1.05	0.78	0.11
(S,R)/(R,R) Fenoterol	0.49	0.13	0.83
(R,S)/(S,S) Fenoterol	3.29	1.63	0.03
(S,R)/(S,S) Fenoterol	1.66	0.26	0.22

^aPreviously reported in reference [9]

^bND = Not Determined