Discovery of Novel Human Phenylethanolamine N-methyltransferase (hPNMT) Inhibitors Using 3D Pharmacophore-Based in silico, Biophysical Screening and Enzymatic Activity Assays

Dong-Ill Kang1, Jee-Young Lee2,3, Woonghee Kim2,3, Ki-Woong Jeong2, Soyoung Shin2, Jiyoung Yang3, Eujin Park5, Young Kee Chae5, and Yangmee Kim2,3,4,*

With the aid of receptor-oriented pharmacophore-based in silico screening, we established three pharmacophore maps explaining the binding model of hPNMT and a known inhibitor, SK&F 29661 (Martin et al., 2001). The compound library was searched using these maps. Nineteen selected candidate inhibitors of hPNMT were screened using STD-NMR and fluorescence experiments. An enzymatic activity assay based on HPLC was additionally performed. Consequently, three potential hPNMT inhibitors were identified, specifically, 4-oxo-1,4-dihydroquinoline-3,7-dicarboxylic acid, 4-[(benzo[d][1,3]dioxol-5-ylamino)-4-oxobutanolic acid, and 1,4-diaminonaphthalene-2,6-disulfonic acid. These novel inhibitors were retrieved using Map II comprising one hydrogen bond acceptor, one hydrogen bond donor, one lipophilic feature, and shape constraints, including a hydrogen bond between Lys57 of hPNMT and a hydrogen bond donor of the inhibitor, and stacked hydrophobic interactions between the side-chain of Phe182 and an aromatic region of the inhibitor. Water-mediated interactions between Asn267 and Asn39 of hPNMT and the amide or amine group of three potent inhibitors were additional important features for hPNMT activity. The binding model presented here may be applied to identify inhibitors with higher potency. Moreover, our novel compounds are valuable candidates for further lead optimization of PNMT inhibitors.

INTRODUCTION

N-methylation is a prominent pathway for the metabolism of several endogenous hormones and neurotransmitters. This reaction occurs via transfer of a methyl group from S-adenosyl-L-methionine (SAM) to nucleophilic amino groups, leading to the production of N-methylated metabolites and S-adenosylhomocysteine (SAH). Adrenaline (or epinephrine) accounts for 5-10% of total catecholamines in the central nervous system (CNS). Adrenaline is synthesized in vivo from noradrenaline in a reaction catalyzed by phenylethanolamine N-methyltransferase (PNMT), a 30 kDa enzyme that utilizes the cofactor SAM to methylate the amine of noradrenaline. PNMT is employed as a catecholamine biosynthetic marker, and the presence of PNMT-containing neurons in the brain suggests that CNS adrenaline is involved in the central control of blood pressure, respiration, and pituitary hormone secretion (Martin et al., 2001). It has been implicated in the effects of ethanol intoxication and neural degeneration observed in Alzheimer’s disease (Kennedy et al., 2004; Meyford et al., 1990). There have been efforts to develop potent PNMT inhibitors as angina pectoris, myocardial infarction and anxiety neuroses agents (Commins, 2001; Križanová et al., 2007).

An extended series of investigations has focused on identifying effective substrates and inhibitors of hPNMT. The majority of these compounds are based on 1,2,3,4-tetrahydroisoquinoline and its analogues (Grunewald et al., 2005a; 2005b; 2006; 2007; 2008; Romero et al., 2004). Receptor-oriented pharmacophore-based in silico screening allows the systematic analysis of possible interactions between a large number of compounds and proteins, leading to the detection of noncovalent interactions in active sites of proteins (Fisher and Güner, 2002; Hoffrén et al., 2001; Kirchmair et al., 2001; 2007; McNnes, 2007). To identify novel and specific ligands, the active sites of proteins are analyzed for establishing pharmacophore maps, which depict sets of interactions (chemical features or functionalities) aligned in three-dimensional space, and include several features, along with excluded volume regions, based on the positions of receptor atoms (Elhallaoui et al., 2002; Pickett et al., 1996). For each library of compounds, a conformationally flexible database is constructed and searched with the set of pharmacophore maps. The resulting hits comprise

Received February 9, 2010; accepted March 9, 2010; published online May 20, 2010

Keywords: adrenaline, in silico screening, PNMT, pharmacophore, STD-NMR
Various conformers of a subset of compounds that satisfy one or more maps, and are thus expected to fit the active site reasonably well.

In our previous study, we performed a docking study for hPNMT and flavonoids, and suggested several interactions between hPNMT and its candidate inhibitors (Lee et al., 2009a). In this study, receptor-oriented pharmacophore-based in silico screening of hPNMT was performed to identify inhibitors of hPNMT. Candidate inhibitors were subsequently assessed for binding to hPNMT using biophysical screening methods, such as STD-NMR and fluorescence experiments. Enzymatic inhibition was further monitored by HPLC.

MATERIALS AND METHODS

Expression and purification of Hpnmt
hPNMT cDNA was a kind gift from the 21C Human Gene Bank, Genome Research Center, KRBIB, Korea. A hexa histidine-tagged hPNMT expression vector, pET-28a-hPNMT-His, was constructed by cloning into the BarnHI/XhoI restriction sites of pET-28a (Novagen, USA), and transformed into E. coli strain BL21. Cells were cultured at 30°C for 5 h prior to harvest by centrifugation, and resuspended in buffer comprising 20 mM Tris-HCl and 500 mM NaCl, pH 7.4 (buffer A). After sonication, centrifugation, and resuspended in buffer comprising 20 mM Tris-HCl and 500 mM NaCl, pH 7.4 (buffer A), hPNMT fraction collected was subjected to gel filtration on a Superdex 75 column (Amersham Biosciences, 5 ml). The column temperature was maintained at 25°C, and a UV detector was monitored at 280 nm. To assess enzymatic inhibition, 125 μl of 20 μM hPNMT and 62.5 μl of 80 μM SAM were mixed thoroughly and incubated for 1 h at 37°C. The hPNMT fraction collected was subjected to gel filtration chromatography on a Superdex™ 75 column (Amersham Biosciences) with 10 mM Tris-HCl, pH 7.0, and collected hPNMT fraction was exchanged into buffer A. Purified hPNMT was identified with 10% SDS-PAGE, and protein purity assessed using MALDI-TOF mass spectrometry.

Building of a 3D compound database
A 3D compound dataset was built with 200,000 synthetic compounds supported by Specs.net (Netherlands). Compounds were converted to 3D multiple conformers by Discovery Studio (DS) Catalyst DB Build module of the DS modeling 2.1 (Accelrys Inc., USA) (Lee et al., 2009a; 2009b; Taha et al., 2007). FAST method was used for multiple conformer generation that allowed 250 maximum conformers, and default values of all other parameters were applied.

Receptor-orientated pharmacophore-based in silico screening of Hpnmt
We defined the active site of hPNMT using the center and radius of the docked inhibitor, which based on the x-ray complex structure of hPNMT and a potent and selective PNMT inhibitor, SK&F 29661 (1HNN.pdb) and determined multiple pharmacophore maps (Martin et al., 2001). A list of features, including hydrogen bond donors (HBDs), hydrogen bond acceptors (HBAs) and lipophilicity (Lipo), were used to determine the pharmacophore map. Maps were generated with the excluded volume for heavy atoms, which is the forbidden area in the active site that defines its shape. To account for excluded volume regions occupied by heavy atoms in the receptor, an exclusion model was generated for the active site and surrounding receptor regions. Each atom of the receptor selected for inclusion in the model was presented as an exclusion point (Hoffrén et al., 2001; Kirchhoff et al., 2001). Among these multiple pharmacophore maps, the most suitable one representing the binding model between hPNMT and inhibitor was established correctly via in silico screening with SK&F 29661. Pharmacophore maps that effectively expressed the binding model of enzyme with its inhibitors were selected for searching the compound library.

Using the final pharmacophore maps, we searched the Specs compound library and selected the candidates of hPNMT inhibitors based on visual inspection and estimation of the ligand score (LigScore) (Apama et al., 2005; Venkatachalam et al., 2003). In particular, shape constraints were applied in database screening for regulating the number of hit compounds. These compounds were further subjected to medium-throughput screening. We performed all computational studies in a Linux environment using the DS modeling/SBP module (Accelrys Inc., USA) (Zou et al., 2008).

HPLC enzymatic inhibition assay
Noradrenaline and adrenaline were separated using a Waters Atlantis™ dC18 4.6 × 250 mm, 5 μm column and Waters 2695 Alliance Separation Module. The mobile phase employed was H₂O:acetonitrile:100 mM ammonium acetate, pH 5.0 = 10:2:88. The column temperature was maintained at 25°C, and a UV detector was monitored at 280 nm. To assess enzymatic inhibition, 125 μl of 20 μM hPNMT and 62.5 μl of 80 μM SAM were mixed thoroughly and incubated for 1 h at 37°C, followed by reaction with a DMSO stock (5 μl of 50 mM) of inhibitors for 15 min at 37°C. Noradrenaline (62.5 μl of 40 μM) was added and incubated for 15 min at 37°C. Reactions were terminated with 20 μl perchloric acid. The supernatant fractions were centrifuged at 6,000 rpm for 5 min, and 10 μl of sample solution was subsequently injected into the HPLC system maintained at 4°C and the chromatogram was monitored for 20 min. Final concentrations of hPNMT, SAM, inhibitor and noradrenaline were 10 μM, 20 μM, 1000 μM and 10 μM, respectively. The inhibition (%) was calculated by monitoring enhancement of the adrenaline peak as follows:

\[
\text{Inhibition} = \left(1 - \frac{\text{integral value of adrenaline of each samples}}{\text{integral value of adrenaline of control sample}}\right) \times 100
\]

A control sample (with 100% of noradrenaline converted to adrenaline) was prepared under similar reaction conditions, but in the absence of inhibitor. The results were compared with those obtained with the commercially available hPNMT inhibitors, THIQ, 2-aminoindan (Grunewald et al, 1981; 2006). The dependence of percentage inhibition on inhibitor concentration was assessed by varying the concentrations of YPN010, YPN016, YPN017, THIQ and 2-aminoindan to 100, 250, 500, 750 and 1000 μM. The time dependence of percentage inhibition was monitored against various reaction times (ranging from 8 to 180 min) with YPN010 and THIQ at a fixed concentration of 500 μM.

NMR screening
Saturation transfer difference (STD) NMR have been widely used for the study of protein-ligand interaction. Information can be gained quickly and easily with these techniques. They need only small amounts of non-isotope-labeled, and thus readily available, target macromolecules (Meyer and Peters, 2003). STD-NMR was performed at KBSI (Korea Basic Science Institute) to identify the inhibitors bound to PNMT. Spectra were collected from regions containing only protein resonances at 298 K, both with and without saturation (Macnaughtan et al.,