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In silico approaches to identify the potential inhibitors of glutamate carboxypeptidase II (GCPII) for neuroprotection

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HIGHLIGHTS

- GCPII inhibition neuroprotective.
- Urea-based NAAG analogue exhibited potential inhibition across all GCPII variants.
- This lead molecule exhibited strong interactions with both the active Zinc ions.
- It satisfied both the Lipinski rule of five and rule of three for drug-likeliness.

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ABSTRACT

To develop a potential inhibitor for glutamate carboxypeptidase II (GCPII) effective against all the eight common genetic variants reported, PyMOL molecular visualization system was used to generate models of variants using the crystal structure of GCPII i.e. 2OOT as a template. High-throughput virtual screening of 29 compounds revealed differential efficacy across the eight genetic variants (pIC_{50}: 4.70 to 10.22). Pharmacophore analysis and quantitative structure-activity relationship (QSAR) studies revealed a urea-based N-acetyl aspartyl glutamate (NAAG) analogue as more potent inhibitor, which was effective across all the genetic variants of GCPII as evidenced by glide scores (-4.32 to -7.08) and protein-ligand interaction plots (13 interactions in wild GCPII). This molecule satisfied Lipinski rule of five and rule of three for drug-likeliness. Being a NAAG-analogue, this molecule might confer neuroprotection by inhibiting glutamatergic neurotransmission mediated by N-acetylated alpha-linked acidic dipeptidase (NAALADase), a splice variant of *GCPII*.

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1. Introduction

Glutamate carboxypeptidase II (GCPII) is a membrane-bound zinc metallo-enzyme that plays a pivotal role in several diseases due to its splice variants, namely prostate specific membrane antigen (PSMA), N-acetylated-alpha-linked acidic dipeptidase (NAALADase), folate hydrolase 1 (FOLH1) (Hlouchová et al., 2007). In the brain, NAALADase is expressed along the surface of astrocytes and catalyzes the hydrolysis of N-acetyl-aspartyl-glutamate (NAAG) to N-acetyl-aspartate (NAA) and glutamate (Rojas et al., 2002). NAAG activates metabotropic glutamate receptor 3 (mGluR3), which are expressed on the surface of presynaptic neurons and astrocytes in the central nervous system. The activation of mGluR3 reduces cAMP and cGMP levels and inhibits release of glutamate. Over expression of NAALADase leads to excitotoxicity and neuronal cell death (Bařinka et al., 2012). PSMA is highly expressed in prostate cancer (Maresca et al., 2009) and in neovasculature of other cancers (Chang et al., 1999). Other splice variant of GCPII i.e. FOLH1 helps in the catabolism of folyl polyglutamate to folyl monoglutamate thereby facilitating the intestinal absorption of folate (Chang et al., 1999). Folate deficiency has been reported to be associated with certain cancers. Thus, GCPII is recognized as a possible drug target in several cancers and various neurological disorders.

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Whole genome sequencing of GCPII has revealed the presence of eight common genetic variants, namely, V108A, P160S, Y176H, R190W, D191V, G206R, G245S, H475Y (Navrátil et al., 2014). Out of which R190W, H475Y, P160S have been shown to confer protection against breast cancer and prostate cancer, whereas D191V and G245S have been reported to be risk factors for the breast and prostate cancer (Navrátil et al., 2014). Studies of GCPII genetic variants in association with neurological diseases are sparse. Earlier, we have reported protective role of GCPII H475Y variant against autism (Divyya et al., 2013). In another study, we have demonstrated the protective role of V108A, P160S and R190W variants against stroke while G245S variant was shown to increase the risk (Divyya et al., 2012). Any compound targeted to inhibit GCPII should be effective against all the eight variants of GCPII.

Various families of potent and selective GCPII inhibitors, such as 2-PMPA, 2-MPPA, and ZJ43 have been developed till date (Divyya et al., 2012; Barinka et al., 2008). Nearly all the potent GCPII inhibitors have zinc-binding group that interacts with the zinc atom present in the active site of GCPII. PMPA based inhibition exhibits higher efficacy towards all the GCPII variants. Despite the efficacy of NAAG and PMPA in inhibiting glutamatergic neurotransmission, the poor oral bio-accessibility of these two compounds restricts their practical use in man. Oral administration of the NAALADase inhibitor GPI-5693 attenuates cocaine-induced reinstatement of drug-seeking behavior in rats (Peng et al., 2010). Stoermer et al. have suggested that phosphinate, urea, phosphonate, thiol, and hydroxamate groups can be effective zinc-binding groups for GCPII inhibitors (Stoermer et al., 2003).

In the current work, we selected 29 compounds belonging to thioalkyl, thiolactone, urea, urea based NAAG analogue series to demonstrate their efficacy in inhibiting GCPII (Stoermer et al., 2012; Ferraris et al., 2014; Tykvart et al., 2014). Sulfhydryl groups present in the thioalkyl and thiolactone compounds tend to form non-covalent bond with the active site of the enzyme, thus contributing to the covalent catalytic activity in catalytic triads. These groups may also respond with heavy metal ions (Zn^{2+}, Cd^{2+}, Pb^{2+}, Hg^{2+}, Ag^{2+}) because of the high affinity between the soft sulphide and the soft metal (Nicholas and Eranthie, 2014). This can deform and inhibit the functional aspects of protein. Also, urea-based compounds make direct interaction by forming hydrogen bonds with polarized areas of core protein, such as peptide groups. This mutual influence weakens the inter-molecular bonds and interactions and disrupts the overall secondary and tertiary structure, thus inhibiting the activity of the target protein molecule.

2. Methods and materials

2.1. Retrieval of protein template and generation of variants

The crystal structure of 2OOT retrieved from Protein Data Bank was used as a template. It has 750 amino acid residues and its active site contains two zinc ions co-ordinated by side chain of His377, Asp387, Glu425, Asp453, His553, which are indispensable for GCPII hydrolytic activity. Wizard Mutagenesis tool of PyMOL molecular visualization system was used to generate eight variants of GCP II (Fig. 1).

2.2. Preparation of ligands

The ligands were prepared by Chemsketch (ACD/Labs) and used for docking studies. These ligands belong to four classes: thiolactones ($n=6$), thioalkyl ($n=4$), urea ($n=16$), urea-based NAAG analogues ($n=3$) (Fig. 2 and 3).

2.3. Docking analysis by high-throughput virtual screening (HTVS)

High throughput screening, docking and scoring techniques were applied to screen all the ligands targeted against GCPII (Vyas et al., 2008). Ligand-based virtual screening was opted to discover the new ligands on the basis of their biological structure. Flexible protein-ligand docking was selected in order to calculate the binding affinity between GCPII variants and 29 inhibitors as this method minimizes the non-specific interactions of the ligand molecule with the protein. Energy functions that evaluate the binding free energies between the protein and the ligand were referred to as “scoring functions”. Based upon these scoring values, ranking of different inhibitors in a dataset was performed through the virtual screening technique.

2.4. Pharmacophore and 2D-QSAR studies using phase

We have used Phase module of Schrodinger for performing pharmacophore studies and QSAR model generation. This module provides support for lead discovery, SAR development, lead optimization and lead expansion (Dixon et al., 2006). All the ligands were converted into 3D-structures and all possible conformers were generated. The IC_{50} values from literature were converted to pIC_{50} using this formula $pIC_{50} = -\log_{10}(IC_{50})$. Based on the active threshold value, 70% of the compounds were considered to be active. And so, the pharmacophore model was produced by using the set of pharmacophore features to create the pharmacophore sites. The phase provides six sets of built-in features: hydrogen-bond donor (D), hydrogen-bond acceptor (A), hydrophobic/non-polar group (H), negatively ionizable (N), positively ionizable (P) and aromatic ring (R) (Rewatkar et al., 2011). Two hydrogen bond acceptors and one hydrogen bond donor were considered as the pharmocophore sites for a given dataset. An identical set of features with similar spatial arrangements were grouped together. Scoring procedure was applied to identify the pharmacophore from each surviving 'n' dimensional box that yields the best alignment of the chosen actives and hypothesis is created (Rong et al., 2002). The test set ($n=9$) and training set ($n=20$) were divided on the basis of structural diversity among the compounds. Based upon this hypothesis 2D-QSAR models were built for all the ligands.

2.5. Model validation through Glide Docking

Docking was performed across the final QSAR model and all the eight variants of GCP II using Glide module of Schrodinger. Flexible ligand docking approach was performed in our study in order to identify the optimal geometry of the ligand and also to minimize the non-specific interactions of ligand molecule. This approach mainly consists of (i) Protein preparation process to optimize the crystal structure of protein by eliminating the overlapping residues; (ii) Ligand preparation step to minimize the ligand molecule; (iii) Receptor grid generation; and (iv) Ligand docking process to know how the ligand interacts with the active sites of the protein molecule. Thus, the binding affinity of QSAR model with the GCPII protein was analyzed through its glide score. Energy and binding affinity were inversely proportional to each other, so more negative the glide score, stronger will be the binding of ligand to protein.

2.6. Predicting the drug likeliness of a model

The drug likeliness of the model was predicted using a Qikprop prediction programme of Schrodinger, which predicts physically significant descriptors and pharmacologically relevant properties of any organic molecules (Jorgensen and Duffy, 2002; Tanwara

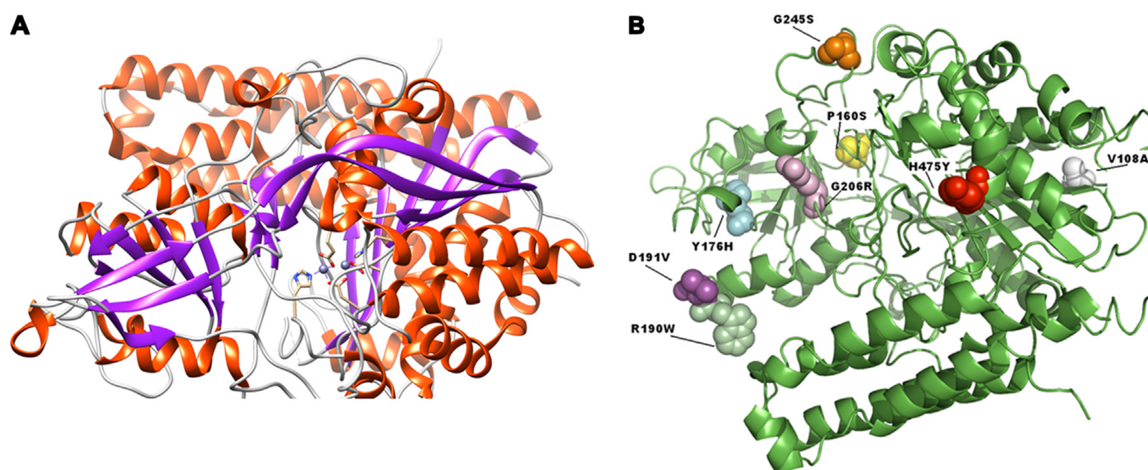

Fig. 1. GCPII protein structure, (A) Crystal structure of glutamate carboxypeptidase II (PDB Id:2OOT), which was used as a template; (B) Structure of glutamate carboxypeptidase II depicting the location of eight common genetic variants, i.e. V108A, P160S, Y176H, R190W, D191V, G206R, G245S, H475Y.

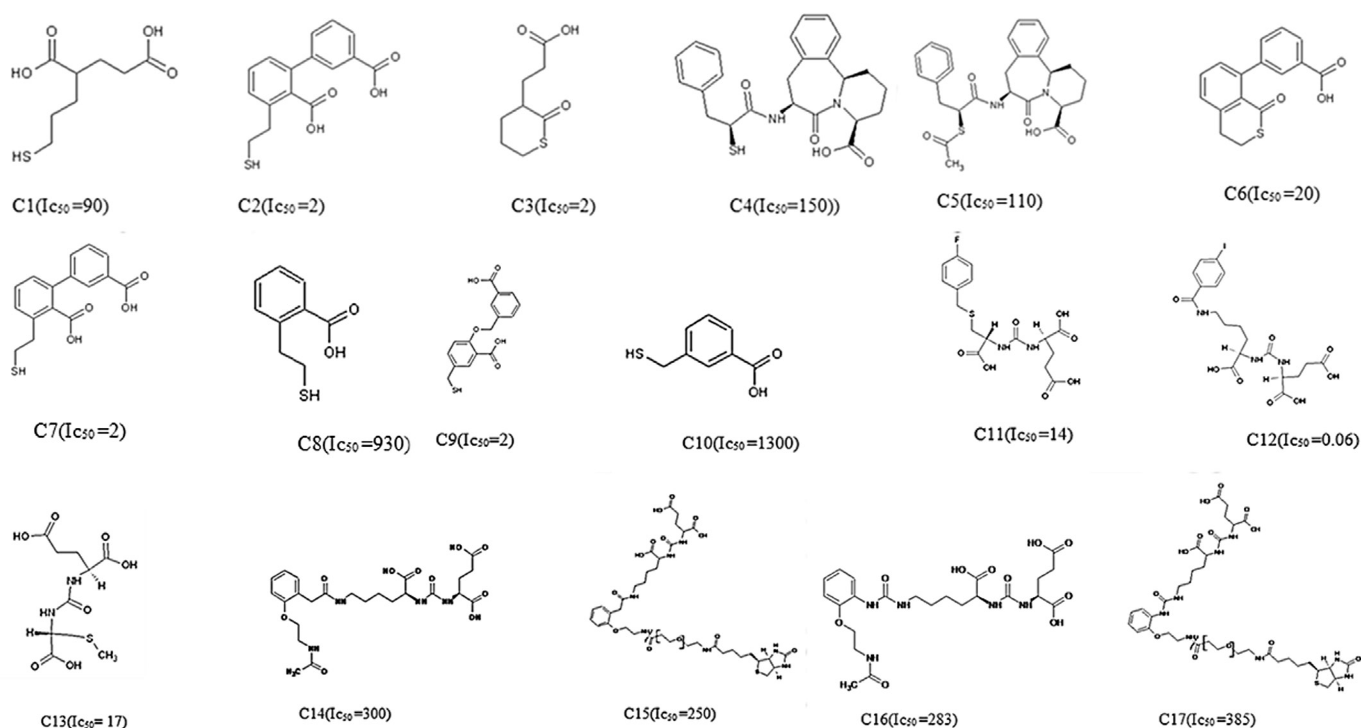

Fig. 2. Chemical structures of various ligands, Using Chem draw, 17 ligand structures, i.e. C1 to C17 were generated to inhibit glutamate carboxypeptidase II. The half maximal inhibitory concentration (IC_{50}) values were depicted for each structure.

et al., 2014). Further, the drug likeliness was assessed based on'Lipinski Rule of Five" and "Rule of Three".

3. Results

HTVS analysis revealed higher affinity of compound 2 towards wild type, V108A, Y176H, P160S, D191V, G206R, R190W variants and lesser affinity towards G245S and H475Y variants. Compound 8 showed an equal affinity towards all the GCPII variants. Compound 16 showed high-degree of variation in affinity profile across different GCPII variants. Compounds 5, 6, 9, 10, 11, 12, 13, 18, 19, 21 and 22 showed no affinity towards any of the GCPII variants. Since we observed variant-specific differential affinity profile with certain variants, pharmacophore analysis and 3-D QSAR was used to develop a potent inhibitor which could inhibit all the GCP II variants. (Supplementary table 1).

Therefore, all the 29 inhibitors were prepared using Ligprep module of Schrodinger. Phase module of Schrodinger generated the three pharmacophore sites namely (acceptor, donor, acceptor). Using these sites, atom based-QSAR modelling approach was opted to generate a QSAR model that inhibits GCP II (Fig. 4). This model has the scaffold of urea-based NAAG analogue and good agreement was observed between actual vs. predicted biological activity. ($R^2=0.99$, $P=5.21\times10^{-15}$). Further, this compound is tested for its drug likeliness.

Lipinski rule states that, drug molecule should possess molecular weight <500; QPlogPo/w <5; donor HB ≤5; accpt HB ≤10 and the Qikprop tool of Schrodinger calculates all the properties together and the recommended range is maximum 4. Also, rule of three describes about, QPlogS >-5.7; QPPCaco >22 nm/s; Primary Metabolites <7 and the recommended range is maximum 3.

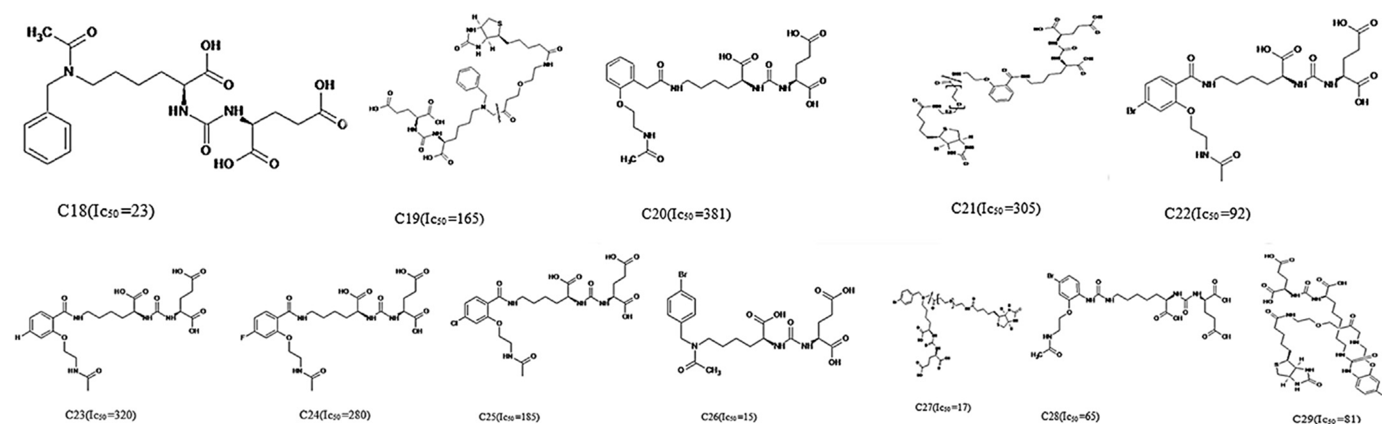

Fig. 3. Chemical structures of various ligands, Using Chem draw, 12 ligand structures, i.e. C18 to C29 were generated to inhibit glutamate carboxypeptidase II. The half maximal inhibitory concentration (IC_{50}) values were depicted for each structure.

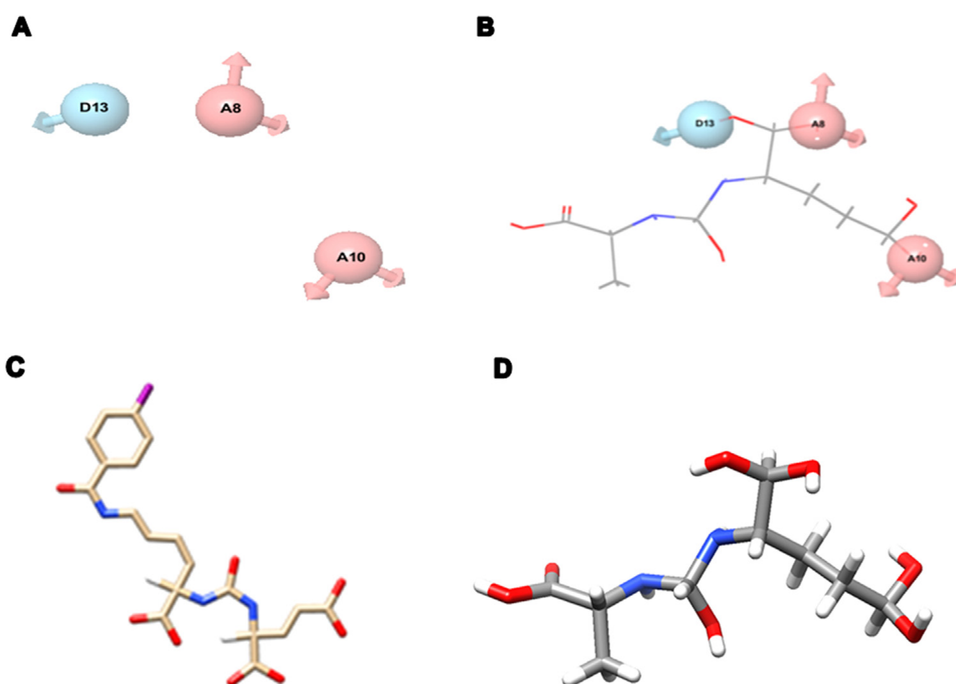

Fig. 4. QSAR model as potential inhibitor of glutamate carboxypeptidase II, (A) Drug-likeliness is dictated by one donor (D13) and two acceptor atoms (A8 and A10); (B) QSAR model; (C) Parent compound; (D) Final drug.

The QSAR model exhibited the range of 1 in both the rules, therefore this model which has a scaffold of urea based NAAG analogue satisfied both “Lipinski Rule of Five and Rule of Three”.

Further, docking studies were performed, and it revealed that this QSAR model showed higher efficacy towards all the variants (Table 1). Protein-ligand interaction plots revealed the interaction of active site zinc ions with the carbonyl group of urea-based NAAG analogue. In most of the variants, Glu424 residue is also interacting with the same carbonyl group of the ligand. (Fig. 5).

Table 1
Glide scores of final QSAR model with all genetic variants of GCPII.

Variants	Glide score(kcal/mole)	Number of interactions with ligand
Wild	−4.31	13
V108A	−5.025	5
P160S	−6.44	10
Y176H	−7.08	6
R190W	−5.25	11
D191V	−6.25	9
G206R	−5.94	8
G245S	−5.39	12
H475Y	−7.02	9

4. Discussion

In the current study, we have screened 29 compounds to test their efficacy in inhibiting GCPII. Certain compounds, although exhibited potency in inhibiting GCPII, they were not effective against all the variants of GCPII. The pIC_{50} values of these compounds ranged from 4.70 to 10.22. A total of 22 compounds showed pIC_{50} of ≥ 6.5 and were considered as active inhibitors of GCPII. Pharmacophore analysis followed by QSAR studies proposed a novel urea-based NAAG analogue as the most potent inhibitor of GCPII. This molecule satisfied Lipinski rule of five and rule of three for drug-likeliness. This molecule was found to have good affinity towards all the variants of GCPII with glide scores ranging from −4.32 to −7.08. This molecule exhibited a total of thirteen interactions with the active site of the wild GCPII.

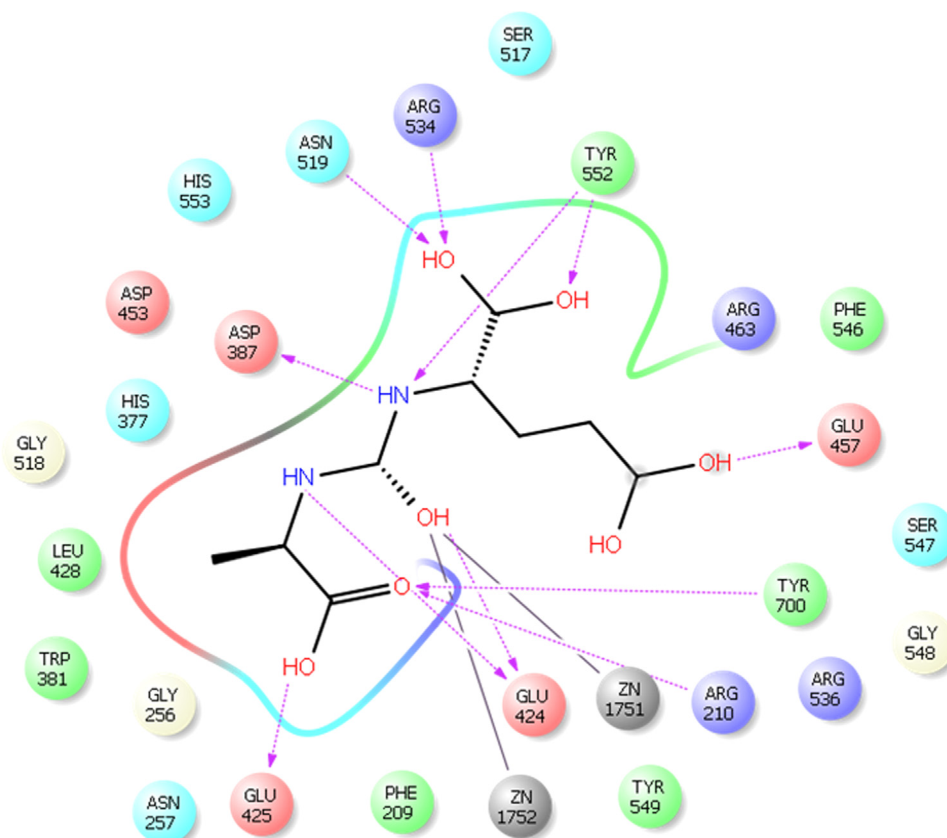

Fig. 5. Interaction of wild type of GCPII with the inhibitor, Two zinc residues of GCPII interacting with carbonyl group of urea-based NAAG analogue. Glu 424 thus acts like a proton shuttle that attracts a proton from the zinc-bound water and donates it to the leaving amino moiety of glutamate during hydrolysis. Most of the interaction of wild type GCPII with the inhibitor are side chain interactions. Dotted lines suggest H-bonding interactions.

The potential application of peptide analogues of NAAG in inhibition of GCPII was demonstrated by earlier studies. These inhibitors possessed a glutamate residue at the C-terminus and had IC_{50} values in nano-molar range. These studies suggested N-acyl (gamma-glutamyl) glutamate as the minimal fragment required for potent binding to GCPII (Mesters et al., 2006; Serval et al., 1990). 2-(phosphonomethyl) pentanedioic acid (2-PMPA), is one of the most potent inhibitor of GCPII known till date (Jackson et al., 1996). It inhibits GCPII activity by chelating an active site zinc ion of GCPII with its phosphonate group. In several animal models, it was shown to inhibit NAALADase activity, thus contributing to increased NAAG levels, which in turn found to be neuroprotective (Tortella et al., 2000). Despite these potential therapeutic effects in experimental models, 2-PMPA applicability as a therapeutic drug was limited due to the poor pharmacokinetic profile of 2-PMPA, which is attributed to the highly polar nature of the molecule. 2-(3-mercaptopropyl) pentanedioic acid (2-MPPA), also known as GPI 5693), which is the first GCPII inhibitor tested in human showing no adverse drug reactions (van der Post et al., 2005). As the drug molecule designed in this study shows good pharmacokinetic profile based on the Lipinski rule of five and rule of three and has affinity towards all the eight common variants of GCPII, it can be explored further in *in vivo* and *in vitro* studies to assess its neuroprotective and anti-cancer properties.

The parent compound initially screened was (S)-2-3-(S)-1-carboxy- (4-iodobenzamido)phenyl- ureido-pentanedioic acid and the final drug designed was (2R)-2-{[S]- hydroxy{[2S)-1,1,5,5-tetrahydroxypentan-2-yl]amino}methyl]amino} propanoic acid (Fig. 4). This molecule forms two covalent bonds with active site zinc ions with the help of OH group on the carbon flanked by NH groups. All other interactions between the protein and ligand are H-bonding interactions. The structural similarity between NAAG and this drug molecule might be inducing competitive inhibition and thus preventing the hydrolysis of NAAG. GCPII inhibition was shown to improve chemotherapy-induced nerve conduction velocity deficits (Wozniak et al., 2012).

Speno et al. suggested that Glu424 residue of GCPII acts like a catalytic acid/base of GCPII. In a free state, the carboxylate 'O' forms H-bond with water molecule bridging the two zinc ions (Speno et al., 1999). Thus Glu424 residue might act like a proton shuttle that attracts a proton from the zinc-bound water and donates it to the leaving amino moiety of glutamate during hydrolysis. In the current study, we have observed this phenomenon as Glu424 is interacting with the same OH group of the drug that forms covalent bonds with zinc ions.

The GCPII-drug interactions as depicted in our study are in agreement with crystal structures of GCPII with potent and weak inhibitors in demonstrating the participation of all the three domains of GCPII namely protease-like, apical and C-terminal in substrate binding (Mesters et al., 2006).

The interaction of drug molecule with protein was found to alter depending on the presence or absence of genetic variants. In wild GCPII, most of the interactions are with side chain residues while in other variants of GCPII, both side chain and backbone interactions were observed. Hence the wild GCPII affinity towards the drug was less (-4.31) compared to GCPII variants (glide score: -5.02 to -7.08).

5. Conclusions

QSAR results have revealed urea-NAAG analogue as the potential inhibitor to demonstrate the efficacy of the drug on all the

genetic variants of GCPII. Further studies on urea-based NAAG analogue will help in demonstrating its neuroprotective role in *in vitro* and *in vivo* models.

Conflicts of interest

All the authors hereby declare no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jtbi.2016.07.016.

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