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Structural characterization of P1′-diversified urea-based inhibitors of glutamate carboxypeptidase II

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ABSTRACT

Urea-based inhibitors of human glutamate carboxypeptidase II (GCPII) have advanced into clinical trials for imaging metastatic prostate cancer. In parallel efforts, agents with increased lipophilicity have been designed and evaluated for targeting GCPII residing within the neuraxis. Here we report the structural and computational characterization of six complexes between GCPII and P1′-diversified urea-based inhibitors that have the C-terminal glutamate replaced by more hydrophobic moieties. The X-ray structures are complemented by quantum mechanics calculations that provide a quantitative insight into the GCPII/inhibitor interactions. These data can be used for the rational design of novel glutamate-free GCPII inhibitors with tailored physicochemical properties.

Urea-based small-molecule inhibitors targeting human glutamate carboxypeptidase II (GCPII) were originally developed for application within the central nervous system (CNS), however, they were first used in vivo to image a peripheral version of GCPII known as the prostate-specific membrane antigen (PSMA) and prostate cancer (PCa).[1–3] GCPII/PSMA (referred to throughout as GCPII) is now a well-established biomarker for imaging PCa, as this membrane-tethered metallopeptidase is over-expressed on the surface of castrate-resistant prostate tumors with its active site facing the extracellular milieu. Additionally, GCPII expression in the neovasculature of most solid tumors, but not normal vasculature, expands the utility of the enzyme for imaging/therapy of tumors other than prostate.[4] In the nervous system, GCPII cleaves *N*-acetylaspartylglutamate (NAAG), releasing *N*-acetylaspartate and glutamate. Excessive glutamate production and release may overstimulate several glutamate receptor subtypes and lead to glutamate-associated neurotoxicity. Furthermore, a glutamate imbalance is linked to the pathophysiology of certain neurological diseases including schizophrenia, amyotrophic lateral sclerosis, neuropathic/diabetic pain and ischemia. Given the involvement of GCPII in a glutamate metabolism, inhibition of GCPII can be used as a therapeutic option for the prevention and treatment of neurological disorders as documented in several animal models of aforementioned diseases.[5]

Imaging agents targeting GCPII can be divided into three categories that include antibodies, aptamers and small-molecule ligands. Currently, only ProstaScint®, an ^{111}In-labeled monoclonal antibody, has been approved by the FDA and is used clinically for imaging PCa, although with variable degrees of success.[6] Because of the long biological half-life of antibodies, leading to excessive non-specific binding, several small-molecule ligands have recently entered clinical trials as viable alternatives for imaging PCa.[7,8] Typically, GCPII inhibitors for imaging applications are derivatives of NAAG with the principal C-terminal (P1′) docking glutamate moiety and the distal (P1) moiety that fine-tunes inhibitor affinity towards GCPII and harbors a radioactive or fluorescent tracer. The P1 and P1′ functionalities are connected via a zinc-binding group (ZBG), resistant to hydrolysis. The most common ZBGs are

Abbreviations: BBB, blood–brain barrier; GCPII, glutamate carboxypeptidase II; NAAG, *N*-acetyl-aspartyl-glutamate; QM, quantum mechanics; PCa, prostate cancer; SAR, structure-activity relationship.

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phosphonates, phosphinates, phosporamidates, and ureas, with the latter being closest to become human medicines.[9–13]

Inhibitors of GCPII that target the CNS or peripheral nervous system, show promise in various animal models of neurological disorders.[14,15] For example, 2-phosphonomethylpropanedioic acid (2-PMPA), a phosphonate-based picomolar GCPII inhibitor, was used successfully to provide neuroprotection following middle cerebral artery occlusion, attenuate cocaine/ethanol-induced drug-seeking behavior, and alleviate hyperalgesia/allodynia in rat pain models.[16–18] The urea-based GCPII inhibitor, ZJ-43, was effective in several pain as well as brain and spinal cord injury models.[18,19] The main pitfall associated with a use of NAAG-based or glutamate-based inhibitors is their high polarity, which stems from the presence of the C-terminal glutamate moiety. In consequence, such inhibitors poorly penetrate the blood–brain barrier (BBB) and their efficacy is limited. Several strategies are being developed to address that problem, including a use of lipophilic prodrugs and the substitution of the P1′ glutamate with a less polar functionality.[20–22]

Recently, we have published a comprehensive study of structure–activity relationship (SAR), in which we described modifications of the urea-based inhibitor, DCIBzL [compound (**7**)], at the P1′ glutamate.[23] The aim of that study was to map the specificity of the S1′ pocket in GCPII towards non-glutamate moieties. We have showed that a variety of isosteres in the P1′ position is tolerated by the enzyme, however, substitution of the C-terminal glutamate inevitably leads to a decrease in inhibitor affinity by several orders of magnitude. Despite that drop in potency, the most potent isosteres still displayed low-nanomolar inhibition constants and were suitable for imaging GCPII-positive peripheral tumors in mice. Retention of high affinity combined with a significant increase in lipophilicity of the new isosteres suggest that further optimization of a functionality placed at the P1′ position might provide BBB-penetrable compounds.

Here, we present the follow-up report, detailing interactions between GCPII and a series of six selected isosteres of (**7**) in the P1′ position. By the combination of X-ray crystallography and quantum mechanics (QM) calculations, we aim to increase an understanding of interactions between non-glutamate moieties in the P1′ position of an inhibitor and the S1′ pocket of the enzyme. We selected six compounds to encompass a diversity of isosteres that span affinities for GCPII across two orders of magnitude (Fig. 1). Included are the most potent isosteres (**6**), K_i = 5.3 nM, and (**1**), K_i = 5.3 nM, representing an unsaturated linear chain and a five-membered heteroaromatic ring, respectively, less active (**4**), K_i = 105 nM, and (**2**), K_i = 85 nM, featuring six-membered aromatic rings, and finally, high nanomolar (**3**), K_i = 318 nM, and (**5**), K_i = 254 nM, three-membered ring compounds. Additionally, we included the parent molecule (**7**), featuring a glutarate moiety at P1′ with K_i = 10 pM. Based on previous SAR and structural reports, all compounds have a P1′ configuration corresponding to L-glutamate, with the exception of (**3**), which has no stereogenic center at the P1′ position. The L-stereoisomers typically bind to GCPII with affinities that are several orders of magnitude higher than their D-counterparts, which are unlikely to generate lead compounds. Additionally, excluded were inhibitors lacking the P1′ side chain altogether (i.e., glycine in the P1′) or missing the α-carboxylate functionality of the P1′ moiety. Interactions between the latter and the guanidinium group of Arg210 from GCPII were shown to be crucial to retain affinity to GCPII in both SAR and mutagenesis studies.[23,24]

Crystallization experiments were carried out using an extracellular part of human GCPII (amino acids 44–750) that was heterologously expressed in *Drosophila* S2 cells and purified to homogeneity according to established protocols.[25] Following the final size-exclusion purification step, the protein was concentrated to 10 mg/mL (in 20 mM Tris–HCl, 150 mM NaCl, pH 8.0), flash-frozen in liquid nitrogen and kept at −80 °C until further use.

Diffracting crystals of GCPII/inhibitor complexes were obtained by first preparing a mixture of GCPII and a given inhibitor [mixing stock solution of GCPII (10 mg/mL) and inhibitor (20 mM) at 9:1 (v/v) ratio] and then mixing the GCPII/inhibitor solution with the same volume of the reservoir solution [33% pentaerythritol propoxylate (Sigma), 1.5% polyethylene glycol 3350 (Sigma), and 100 mM Tris–HCl, pH 8.0]. Crystals were grown using the hanging-drop vapor-diffusion setup at 293 K and diffraction intensities for each complex were collected from a single crystal at 100 K using synchrotron radiation at the SER-CAT beamlines 22-ID and 22-BM at the Advanced Photon Source (Argonne, USA). For each complex, a complete dataset was collected from a single crystal and data were processed using the HKL2000 software package.[26] Difference Fourier methods were used to determine structures of GCPII/inhibitor complexes with the ligand-free GCPII structure (PDB code 2OOT)[27] used as a starting model. Calculations were performed using the program Refmac 5.5.[28] and the structural refinement was interspersed by manual corrections to the model with aid of the program Coot 0.6.[29] The PRODRG server was used to generate the restrains library and the coordinate files for individual inhibitors.[30] Detailed procedures, the data collection and refinement statistics are summarized in Supplementary Table S1. Atomic coordinates of the present structures together with the experimental structure factor amplitudes were deposited at the RCSB Protein Data Bank under accession numbers shown in Figure 1.

Structures of GCPII/inhibitor complexes were refined at the resolution range between 1.65 and 1.85 Å, with suitable crystallographic parameters (Table S1). The overall fold of the protein component is nearly invariant as reflected by the maximum root mean square deviation of 0.16 Å for the 683 equiv Cα pairs between GCPII/(**1**) and GCPII/(**2**) complexes. During the final stages of refinement, inhibitors were modeled into the well-defined *Fo–Fc* positive density peaks with high confidence for all six inhibitors (Fig. 1).

The 4-iodobenzoyl-ε-lysine, which is derived from the parent DCIBzL (**7**) molecule, is a structural motif common to all inhibitors presented here. This motif was included in the inhibitor design as its addition increases the affinity of a given compound nearly ten-fold compared to urea-based scaffolds lacking this functionality.[31] That effect results primarily from the engagement of the terminal 4-iodobenzoyl group with an S1 hydrophobic 'accessory pocket' of GCPII shaped by the side chains of Glu457, Arg463, Asp465, Arg534, and Arg536. As expected, all structures feature the 4-iodobenzoyl group inserted into the pocket. The benzene ring of the inhibitor is parallel to guanidinium groups of Arg463 and Arg534, highlighting the importance of π-cation interaction for the inhibitor binding.

The lysine linker connecting the distal 4-iodobenzoyl functionality to the urea isostere is somewhat flexible, yet its conformation is constrained by the invariant positioning of the 4-iodobenzoyl group at one end and the urea at the other. Moreover, the P1 carboxylate group forms hydrogen bonds with side-chains of Asn519 (2.9 Å; in GCPII/(**6**) complex), Arg534 (2.9 Å), and Arg536 (2.9 Å and 3.0 Å), adding additional constraints. The P1 carboxylate has been reported to be an important signature of the NAAG-based inhibitors as its absence or substitution results in a weaker binding to GCPII.[23,32]

Similar to structures of urea-based inhibitors described previously,[31,33] the ureido group of the six inhibitors studied here mimics a planar peptide bond of a GCPII substrate and interacts with several residues in the active site. The ureido carbonyl oxygen is polarized by the catalytic Zn ion (2.7 Å) and also interacts with side chains of Tyr552 (OH, 2.6 Å) and His553 (Nε2, 3.2 Å), and with the hydroxide anion (2.9 Å). The N1 and N2 ureido nitrogen atoms