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Novel substrate-based inhibitors of human glutamate carboxypeptidase II with enhanced lipophilicity

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Abstract

Virtually all low molecular weight inhibitors of human glutamate carboxypeptidase II (GCPII) are highly polar compounds that have limited use in settings where more lipophilic molecules are desired. Here we report the identification and characterization of GCPII inhibitors with enhanced lipophilicity that are derived from a series of newly identified dipeptidic GCPII substrates featuring non-polar aliphatic side chains at the C-terminus. To analyze the interactions governing the substrate recognition by GCPII, we determined crystal structures of the inactive GCPII(E424A) mutant in complex with selected dipeptides and complemented the structural data with quantum mechanics/molecular mechanics calculations. Results reveal the importance of non-polar interactions governing GCPII affinity towards novel substrates as well as formerly unnoticed plasticity of the S1' specificity pocket. Based on those data, we designed, synthesized and evaluated a series of novel GCPII inhibitors with enhanced lipophilicity, with the best candidates having low nanomolar inhibition constants and clogD > -0.3. Our findings offer new insights into the design of more lipophilic inhibitors targeting GCPII.

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PDB ID codes: Atomic coordinates of the present structures together with the experimental structure factor amplitudes were deposited at the RCSB Protein Data Bank under accession numbers 3SJX [GCPII(E424A)/NAAM], 3SJG [GCPII(E424A)/7S], 3SJE [GCPII(E424A)/8S], and 3SJF [GCPII/9I].

Supporting Information Available: Details of data collection and refinement statistics, spectroscopic data for inhibitors, and QM/MM calculations together with resulting PDB files. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

PSMA; NAALADase; GCPII; zinc peptidase; folate hydrolase; inhibition; quantum mechanics/molecular mechanics (QM/MM)

1. Introduction

Human glutamate carboxypeptidase II (GCPII) is a transmembrane metallopeptidase with the expression pattern restricted primarily to nervous and prostatic tissues.¹⁻³ As the expression levels of the prostate form of the enzyme are highly elevated in prostate carcinoma and its metastases, GCPII serves as a membrane-bound marker for prostate cancer imaging and experimental therapy.⁴⁻⁸

Within the nervous system, GCPII catabolizes *N*-Ac-Asp-Glu (NAAG), the dipeptidic neuropeptide, liberating glutamate into the extrasynaptic space.¹ Both NAAG and glutamate act as potent neurotransmitters and their extracellular concentrations must be tightly regulated to secure normal brain functioning. Not surprisingly, dysregulated NAAG (and glutamate) metabolism and signaling are associated with a number of neurological disorders.⁹ Given the intimate involvement of GCPII in NAAG (glutamate) metabolism, the modulation of its enzymatic activity by small-molecule ligands is considered a viable option for the treatment and diagnosis of a variety of pathologies that involve glutamatergic transmission. Indeed, GCPII inhibitors have demonstrated efficacy in experimental models of stroke,¹⁰ diabetic neuropathy,¹¹ amyotrophic lateral sclerosis,¹² neuropathic and inflammatory pain,¹³⁻¹⁵ schizophrenia¹⁶ and drug addiction.^{17, 18} Additionally, radiolabeled GCPII-specific ligands were used to visualize quantitatively GCPII in rodent and human tissue *ex-vivo*,^{19, 20} expanding thus their use into the diagnostic area.

Effective targeting of GCPII residing in nervous tissue requires inhibitors that can penetrate into the neuronal compartment. That can be achieved for example by designing inhibitors that can leverage active transport mechanisms or by using lipophilic compounds with enhanced penetration across the blood-brain barrier. As the early efforts in identifying low molecular weight GCPII inhibitors relied mainly on the knowledge of GCPII substrate specificity, the two major categories of GCPII-specific inhibitors that exist at present are either analogs of NAAG (the GCPII substrate) or derivatives of glutamic acid (the reaction product). Consequently, nearly all currently used GCPII inhibitors are highly polar with their total molecular charges under physiological pH conditions ranging typically between -2 and -3 and with lower likelihood CNS penetration. The design of GCPII-specific inhibitors with increased lipophilicity/hydrophobicity is therefore highly desirable, with the ultimate goal of improving bioavailability and tissue penetration while maintaining potency and specificity for the use in clinical practice.

In our previous work, we reported the analysis of the substrate specificity of human GCPII, and in addition to the known GCPII substrates featuring C-terminal glutamate, we identified P1' methionine as a residue effectively recognized by the enzyme.²¹ Taking into the account the substitution of the glutamate negatively charged side-chain by the more hydrophobic side chain of methionine, we hypothesized that in addition to salt bridges and/or hydrogen bonds, non-polar interactions at the S1' site of GCPII are likely to play an important role in the positioning of the C-terminal amino acid residue of GCPII substrates. These arguments are also consistent with our other findings concerning the importance of specific amino acid residues in the GCPII S1 and S1' sites,²² the detailed study of GCPII reaction mechanism²³ as well as with previously reported series of GCPII-specific inhibitors.^{7, 24-28}

The aim of this work is to explore new directions in the rational design of GCPII inhibitors by increasing their lipophilicity and explain in detail the observed non-polar interaction patterns. To achieve this goal we use a combination of experimental and theoretical approaches including X-ray crystallography, site-directed mutagenesis, and hybrid quantum mechanical and molecular mechanical (QM/MM) calculations. Starting from the detailed characterization of GCPII-methionine interactions within the S1' pocket, we designed and characterized novel GCPII substrates featuring aliphatic side chains at the C-terminal amino acid. Finally, based on our kinetic data, we synthesized and evaluated a series of novel substrate-based GCPII inhibitors with enhanced lipophilicity.

2. Results

Novel GCPII substrates: Ac-Asp-Ala Ac-Asp-Ano series

Since dipeptides with the C-terminal methionine, but not with branched hydrophobic amino acids such as valine or leucine, are efficiently hydrolyzed by human GCPII^{21, 29} we hypothesized that the enzyme might be able to process dipeptides bearing an unbranched, aliphatic side chain at the C-terminal moiety. To test this prediction, we analyzed the hydrolysis of a series of eight dipeptides by recombinant human GCPII (rhGCPII; Table 1). The series was chosen to examine systematically the effect of P1' aliphatic side chain length on the hydrolysis by rhGCPII. The substrate cleavage was followed by high-performance liquid chromatography (HPLC) and the results are summarized in Table 1. Except for **1S** (Ac-Asp-Gly), a substrate lacking a side-chain at the C-terminal amino acid, all other dipeptidic substrates tested were hydrolyzed by rhGCPII. The least efficient substrate in this series was **2S** (Ac-Asp-Ala), *i.e.* the substrate with the shortest amino acid side chain, and gradual extension of the hydrocarbon side-chain of the C-terminal amino acid resulted in the monotonic improvement of the overall catalytic efficiency. This trend is documented by the fact that compared to Ac-Asp-Ala, the rhGCPII hydrolysis of **8S** (Ac-Asp-Ano), the dipeptide with the longest (heptyl) C-terminal side-chain, is approximately 20-fold more efficient (Table 1).

rhGCPII(E424A)/substrate complexes

To elucidate structural features that govern interactions between GCPII and non-polar side chains of P1' residues, we determined X-ray structures of the inactive rhGCPII(E424A) mutant²³ in the complex with three of the biochemically characterized substrates – Ac-Asp-Met (**NAAM**), **7S** (Ac-Asp-Aoc), and **8S** (Ac-Asp-Ano) at resolution of 1.66 Å, 1.65 Å, and 1.70 Å, respectively. (Note: Glu424 acts as a proton shuttle during substrate hydrolysis by GCPII and as such it is indispensable for the enzymatic activity of the enzyme. By mutating Glu424 to alanine we constructed the inactive GCPII(424A) mutant that cannot hydrolyze cognate substrates and serves thus as an excellent tool for elucidating/approximating enzyme-substrate interactions.). All three structures were determined using difference Fourier methods and the refinement statistics of the final models are summarized in the Supplementary table S1. The overall fold of the rhGCPII(E424A) protein in individual complexes is nearly identical to the arrangement observed for the rhGCPII(E424A) complex with NAAG, a natural GCPII substrate reported earlier (PDB code 3BXM).²³ The only major structural deviations in the substrate binding cavity are observed for the Lys699 side chain that comes into contact with the side chains of C-terminal amino acids of novel substrates. The superposition of the active site-bound substrates in the S1' pocket and their comparison to the rhGCPII(E424A)/NAAG complex are depicted in Figure 1.

Substrate orientation in the GCPII binding pocket

Positioning of all three dipeptides within the GCPII binding pocket can be unambiguously assigned from the electron density map and conforms to a canonical model, where the S1

pocket of GCPII is occupied by the acetyl-aspartyl moiety and the C-terminal part of a substrate extends into the S1' site. Even though an equimolar mixture of (1'-*R,S*)-diastereomers was used in the case of **7S** and **8S** dipeptides, only the 1'-*S*-stereoisomers are observed in the GCPII complexes (Figure 1). That observation is consistent with known preferences of GCPII towards L-amino acids in the P1' position of substrates and (*S*)-stereoisomers of inhibitors.^{1, 30}

The S1' pocket interactions

The enzyme-substrate interactions within the S1' site include both polar and non-polar interactions. The arrangement of polar interactions is analogous to the polar contacts reported earlier for the rhGCPII(E424A) complex with NAAG²³ and includes direct hydrogen bonding/ionic interactions between the C-terminal α -carboxylate and side chains of Arg210, Tyr700, and Tyr552 as well as several water mediated contacts. On the other hand, two hydrogen-bonding interactions between the glutamate γ -carboxylate and the Asn257 and Lys699 side chains are lost in the case of dipeptides with P1' non-polar side chains. Instead, the positioning of the C-terminal aliphatic side chain relies mainly on non-polar contacts with the side chains of Phe209, Asn257, Leu428, Lys699, and main chains of Gly427 and Gly517.

The flexibility of the Lys699 side-chain shapes the S1' pocket

Extended C-terminal side-chains of several novel substrates (the most prominently **7S** and **8S**) are too long (~8.5 Å from C $_{\alpha}$ to the terminal aminononanoic side-chain carbon atom in the extended conformation) to fit conveniently into the standard S1' pocket observed repeatedly for glutamate-like moieties. Instead, the S1' pocket has to be reshaped to accommodate such residues. Our structural data show that the change in size and shape is realized by the out-swing of the Lys699 side chain with the positional shift of the N $^{\epsilon}$ by 3.9 Å. Simultaneously, the C-terminal side-chains adopt a bow-shaped conformation to fill-in the S1' pocket (Figure 1). Clearly, the flexibility of the Lys699 side-chain is crucial for alleviating steric crowding imposed by the presence of a bulkier substrate/inhibitor moiety, contributing thus prominently to the plasticity of the S1' pocket.

To verify the “steric crowding” hypothesis, we constructed a GCPII(K699S) mutant, where Lys699 is mutated to serine. Compared to wild-type GCPII, the K699S mutation results in approximately 3-fold stronger binding of **8S** as determined by the kinetic assay (Table 2). This data suggest that the short side-chain of Ser699 is more accommodating towards the bulkier C-terminal side chains of a substrate than the long Lys699 side-chain. On the other hand, much lower affinity of NAAG towards the K699S mutant (~30-fold increase in the Michaelis constant compared to the wild-type enzyme) confirms the importance of the Lys699-Glu gamma-carboxylate salt bridge for binding of compounds featuring C-terminal glutamate.

Substrate-based inhibitors

Based on our kinetic and structural data, we designed and evaluated a series of novel inhibitors, where the N-Ac-Asp moiety and the peptide bond of a substrate are replaced by the (4-iodo-benzoylamino)hexanoyl functionality and the non-hydrolyzable urea surrogate, respectively. The rationale for the N-Ac-Asp to (4-iodo-benzoylamino)hexanoyl substitution was driven by our prior observation that the incorporation of this functionality into the glutamate-urea scaffold increases the affinity of resulting compounds by several orders of magnitude.²⁴ Inhibitory properties of the novel compounds were determined using the Amplex Red assay and the results are summarized in Table 3. The K_i values in the series follow the general trend observed for the parent substrates, with the inhibitor potency increasing with the elongation of the P1' side chain. In this series, the compound **1I** has the

lowest affinity towards GCPII ($K_i = 4390$ nM), while the inhibition constants monotonically decrease from **1I** through **6I** and plateau for the compounds **6I** – **8I**, reaching low nanomolar affinity (~ 20 nM). The “plateau effect” observed for the inhibitor series mirrors results from the kinetic measurements, pointing towards identical/similar positioning of P1' moieties of substrates/inhibitors. As a result, structural/biochemical observations for one type of ligands, substrate or inhibitor, can likely be extrapolated to the corresponding counterpart and exploited for the design of substrate-based inhibitors in general.

GCPII/9I complex: X-ray structure

To confirm the assumption that binding modes to an inhibitor and its parent substrate by GCPII are similar, we determined an X-ray structure of GCPII in complex with **9I**, a urea-based inhibitor derived from NAAM at 1.65 Å resolution. The binding mode of **9I** in the GCPII specificity pockets is unambiguously defined by the *Fo-Fc* omit map (Figure 2) and mirrors the orientation and positioning of **10I** (DCIBzL; a urea-based compound featuring C-terminal glutamate). More importantly, though, the C-terminal methionine in the GCPII/**9I** complex (together with surrounding GCPII side chains) spatially overlaps with the corresponding part of **NAAM**, its parent substrate (Figure 3). Taken together, these data suggest transferability of kinetic/enzymatic data into the inhibitory profiles of daughter compounds.

GCPII/ligand complexes: QM/MM modeling

Based on the above described crystallographic data, we carried out the QM/MM calculations to extend the experimental structural information over additional aspects of the ligand-GCPII binding such as the protonation equilibria between protein and ligand or energies associated with the [enzyme...ligand] interaction. In total, seven structures were modeled and examined, including complexes of **NAAM**, **7S**, **8S** with both wild-type GCPII and the GCPII(E424A) mutant, as well as the complex of **8I** and **10I** with wild-type GCPII. Moreover, we have considered the protonation of the Glu424 residue (that acts as a proton shuttle in the GCPII catalytic cycle), which resulted in a total of thirteen systems that were studied. A fairly large quantum region (~ 350 atoms) and a neighboring part of the protein optimized at the MM level (additional 110 amino acid residues) should allow for a sufficient degree of flexibility in the binding site.

The resulting optimized structures can be found in the Supplementary material (as PDB files including partial charges on all atoms). The excellent structural agreement between the equilibrium QM/MM structures and their crystallographic counterparts allowed more detailed energy analysis of the interactions between substrates/inhibitors and GCPII (vide infra). Moreover, the QM/MM calculations yielded the equilibrium structures of the wild-type GCPII with **NAAM**, **7S**, **8S** and the model structure of the GCPII complex with **8I** (i.e., structures that have not been determined experimentally), which add to the already extensive structural information obtained in this study. The alignments of the calculated and experimental structures are depicted in Figures S1a-c (Supplementary Material), whereas the newly predicted structures are depicted in Fig. S2.

The most notable structural features of the QM/MM optimized structures not attainable from the X-ray crystallography can be summarized as follows:

1. (1) In wild-type complexes, a hydroxide anion, not a water molecule bridges the two zinc ions in the active site, since the water molecule spontaneously deprotonates upon binding to the $(\text{Zn})_2$ site. Most likely, the proton resides on the Glu424 moiety and is ready for the second step of the peptide bond cleavage as shown in the previous study.²³

2. In the complexes with the non-polar side chains at the C-terminus, the charge of the Lys699 residue which is otherwise the key residue for the interaction with (and the recognition of) the C-terminal (P1') glutamate in GCPII natural substrates is attenuated by a chain of three water molecules that are strongly polarized by the interaction with the charged NH_3^+ group with the O-H bonds elongated by 0.1-0.2 Å in comparison with their standard values.
3. In QM/MM optimized structures, we have not observed any pronounced structural changes related to the protonation of the Glu424 residue as the extra "catalytic" proton²³ is not directly involved in any hydrogen bond. Neither have we noticed any significant effect of the E424A mutation on the overall constitution of the GCPII active site and positioning of the substrate. The superpositions of the wild-type GCPII, GCPII(E424A), and protonated wild-type GCPII structures are given in the supplementary material (Figs. S3-S5).

Interaction energies of non-polar side chains with the S1' site residues

We utilized the results of the QM/MM calculations to provide semiquantitative arguments to the discussions concerning the origin of the non-polar interactions between ligands and the S1' site of GCPII. We have calculated interaction energies of the model ligands representing the side chains of Met, 2-aminooctanoic (AOC), and 2-aminononanoic (ANO) acids with the small models of interacting residues. The system is depicted in Figure 4 and the results are summarized in Table 4. It must be emphasized that the results are only semiquantitative, due to the simplicity of the model functional groups representing the residues, but a few observations can be made.

Concerning the overall value of the interaction energy, one may observe that for ANO and AOC side chains it is marginally higher (0.2-0.4 kcal.mol⁻¹) than for the Met side chain. It qualitatively correlates with the observed decrease in K_M values (**NAAM** vs. **7S** and **8S**). However, these differences are very small and admittedly, the same correlation does not extend to the **7S** vs. **8S** comparison.

In the case of methionine, more than 40% of the overall interaction energy (-11.1 kcal.mol⁻¹) with the neighboring residues comes from the interaction with Asn257 side chain (-4.8 kcal.mol⁻¹). Other non-polar residues contribute by ~ -1.5 kcal.mol⁻¹ per residue with the exception of the non-polar part of the Lys699 side chain (modeled as $\text{CH}_3(\text{CH}_2)_2\text{CH}_3$) that contributes negligibly. For the C-terminal AOC and ANO, there is a notable increase in the interaction of Phe209 and the non-polar part of Lys699 (by ~ 1 kcal/mol⁻¹) and a slight increase in the interaction energies of other non-polar residues that more than compensates the energetic loss in the interaction of AOC/ANO with the Asn257. The same stabilizing role can be also postulated for several of our inhibitors previously published and highlights the importance of π - π stacking interactions in biological systems.

Finally, we observed that the interaction energies between the P1' side chain of the substrate/inhibitor and the S1' residues are almost perfectly pairwise additive, i.e. the total interaction energy almost equals the sum of pair interaction energies. In summary, these calculations provide semiquantitative insight into the arguments about the origin of the hydrophobicity of the S1' site, given in this study.

3. Discussion

Glutamate-based functionalities are instrumental for selective targeting of human GCPII in applications ranging from prostate cancer (PCa) imaging to the experimental treatment of neurodegenerative conditions.⁸ Since the GCPII pharmacophore (S1') pocket is "optimized"

for glutamate-like scaffolds, the presence of these functionalities assures both high affinity and specificity of corresponding inhibitors.^{31, 32} Several groups reported structure-activity relationship (SAR) studies focusing on substituting the P1' glutamate in GCPII inhibitors. Majer *et al*³³ designed and tested a series of thiol-based inhibitors containing a benzyl moiety at the P1' position to increase lipophilicity of 2-(3-mercaptopropyl)pentanedioic acid (2-MPPA), the first orally available GCPII inhibitor. In addition to higher lipophilicity, the best candidates were found to be more potent than the parent molecule and showed effectiveness in rat chronic constriction injury model of neuropathic pain. The Kozikowski group³⁴ applied the SAR approach using *N*-[[[(1*S*)-1-Carboxy-3-methylbutyl]amino]carbonyl]-L-glutamic acid (ZJ-43, a urea-based NAAG analogue as a lead compound) to decrease polarity for more efficient targeting of GCPII in the nervous system, especially in the PNS. We evaluated a series of DCIBzL-based isosteres and identified several non-glutamate inhibitors with K_i values below 20nM that exhibited selective binding to GCPII-expressing tumors by single photon emission computed tomography (SPECT-CT) imaging in mice.³²

This report uses the rational design to extend and complement the above-mentioned SAR studies with the objective of preparing potent GCPII inhibitors with enhanced lipophilicity. Based on our earlier kinetic data we first designed and characterized a set of novel dipeptidic GCPII substrates and provided the structural evidence for recognition of such dipeptides by GCPII. Next, we designed a series of inhibitors, where the P1' moiety is derived from the dipeptidic substrates and the P1 part features 4-iodo-benzoyl- ϵ -lysine, the functionality shown by us previously to augment interactions with GCPII²⁴; the P1 and P1' parts are connected *via* a urea linker (Table 3). The most potent molecule (compound **8I**) has $K_i = 29$ nM and ClogD = -0.23. Although the binding affinity of **8I** is markedly lower compared to the parent glutamate-based compound (29 nM vs 10 pM, respectively), its affinity is sufficient for imaging PCa.³² Furthermore, substantially increased lipophilicity (-0.23 vs -5.16) can be translated into a better pharmacokinetic profile in the periphery, with increased likelihood of the penetration into the CNS. Last but not least, in the phase I human clinical trial using *N*-[*N*-[(*S*)-1,3-dicarboxypropyl]carbamoyl]-4-[¹⁸F]fluorobenzyl-L-Cysteine ([¹⁸F]DCFBC),³⁵ one of the glutamate-based PET agents targeting GCPII that was developed for prostate cancer imaging, we observed somewhat increased signal from the blood pool in human subjects, suggesting potential binding of the compound to an unidentified plasma protein. The prime suspect in the case is plasma glutamate carboxypeptidase, a circulating plasma protein with 27% overall sequence identity and overlapping substrate specificity to GCPII.^{36, 37} Given the substitution of glutamate by non-natural amino acids in novel inhibitors presented here, the likelihood of off-target interactions with endogenous proteins might be less pronounced in the latter. To prove these assumptions, however, additional *in vivo* studies are needed.

For both substrate and inhibitor synthesis, 2-amino acids with pentyl to heptyl side chains were used as a racemic mixture for both substrate and inhibitor synthesis. The corresponding products (substrates and inhibitors) are therefore equimolar mixtures of two diastereomers. In the case inhibitors, the individual diastereomers were separated by HPLC and their inhibition potency assayed. As expected, only compounds with the (*S*) stereochemistry at the C-terminus were inhibitory, while their (*R*) counterparts turned out to be inactive (data not shown). In the case of substrates, a mixture of diastereomers was used for kinetic studies. Following the substrate incubation with rhGCPII for 24 hours at 37°C, we analyzed the reaction mixture using HPLC with UV detection after pre-column derivatization of released C-terminal amino acids with the Marfey's reagent (1-fluoro-2-4-dinitrophenyl-5-L-alanine amide), a chiral reagent used for distinguishing (*S*)- and (*R*)-amino acids. In all cases, we observed peaks corresponding to only a single, presumably (*S*), enantiomer (data not shown). Since previously reported data suggested that GCPII is inactive towards (*R*)-amino

acids at the P1' position, we concluded that only (*S*)-amino acid-containing dipeptides serve as efficient substrates of rhGCPH.

The SAR studies suggest that the non-prime GCPH specificity pocket(s) are rather insensitive to structural changes of GCPH inhibitors and can accommodate (or at least tolerate) surprising diversity of functional groups of inhibitors.^{4, 7, 24, 26, 28, 34} On the contrary, the S1' (or pharmacophore) pocket in GCPH is highly selective for glutamate and glutamate-like moieties. The selectivity is achieved *via* an intricate network of mostly polar interactions between GCPH and an inhibitor, with the most prominent being the ion pairing between Arg210- α -carboxylate and Lys699- γ -carboxylate.^{22, 31} Structural data that characterize the S1' pocket as fairly compact, small-sized and unyielding, in contrast to the much larger and quite flexible non-prime site (the funnel emanating from the active site zinc to the surface of the protein), are in agreement with these observations.

This report expands the above concept in two ways: (i) it documents for the first time substantial plasticity of the GCPH pharmacophore pocket achieved by the relocation of the Lys699 side chain leading to the considerable enlargement (by 3.9 Å) of the S1' site. Furthermore, this work directly demonstrates the importance of non-polar interactions, mediated by the side chains of Phe209, Asn257, Leu428, and Lys699 for GCPH affinity towards small-molecule compounds featuring hydrophobic moieties in the P1' position. Although ionic interactions between the Arg210 guanidinium group and the α -carboxylate group of the C-terminal (P1' position) residue are common to all dipeptidic substrates tested in this study, these are obviously not sufficient with respect to efficient substrate positioning and subsequent hydrolysis, as the dipeptide with glycine in the P1' position is not cleaved. Additionally, the dipeptide with a C-terminal alanine, the amino acid with the shortest side-chain, in the P1' position is the least efficient GCPH substrate (see Table 1).

In summary, the new findings presented here expand the chemical space that can be explored during the rational design of GCPH inhibitors with increased lipophilicity. By linking a lipophilic “non-glutamate” C-terminal moiety to a non-polar P1 functionality one can design inhibitors with increased lipophilicity that are more likely to penetrate the blood-brain barrier. It should be noted, however, that lipophilicity is only one of physicochemical parameters related to the drug-like molecular properties (others being e.g. molecular weight, polar surface area, number of hydrogen bond donors/acceptors, number of rotatable bonds). In this regard, compounds presented here can be viewed as a precedent for the development of GCPH-specific inhibitor analogs, with the ultimate goal of designing the truly BBB-permeable compounds.

4. Conclusions

In this study we i) report the design and characterization of a novel set of dipeptidic GCPH substrates; ii) provide structural and computational evidence for recognition of such dipeptidic substrates by GCPH; and, iii) report the design and evaluation of novel substrate-based GCPH inhibitors with nanomolar affinity and increased lipophilicity. Besides contributing to the understanding of GCPH function, these data also serve as a starting point for the design of “non-glutamate” small molecule GCPH ligands with the increased lipophilicity that may represent a novel and important class of inhibitors of GCPH.

5. Experimental Section

Wild-type rhGCPH Expression and Purification

The cloning, expression, and purification of recombinant human GCPH (rhGCPH) has been described previously.²¹ Briefly, the extracellular part of human glutamate carboxypeptidase

II, which spans amino acid residues 44 to 750, was cloned into the pMT/BiP/V5-His A plasmid (Invitrogen, Carlsbad, USA) and the recombinant protein (designated rhGCPII) was expressed in *Drosophila* Schneider's S2 cells and purified to homogeneity.

Expression and Purification of rhGCPII(E424A) and rhGCPII(K699S) Mutants

Cloning, expression and purification of the rhGCPII(E424A) and rhGCPII(K699S) mutants has been described elsewhere.^{22, 23} In short, desired mutations were introduced into a GCPII coding sequence using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) and the mutated protein expressed in S2 cells. Purification protocols included ion-exchange chromatography steps (QAE-Sephadex A50, Source S15) followed by an affinity chromatography on Lentil-Lectin and a size-exclusion chromatography on a Superdex 200 column. The final protein preparations were >95% pure as determined by silver-stained SDS-PAGE (data not shown). For crystallization experiments, purified rhGCPII(E424A) was dialyzed against 20 mM MOPS, 20 mM NaCl, pH 7.4 and concentrated to 8.7 mg/ml.

Crystallization and Data Collection

The rhGCPII(E424A) stock solution was mixed with 1/10 volume of 100 mM aqueous solutions of **NAAM**, **7S**, and **8S**. For the GCPII/**9I** complex, rhGCPII stock solution (8 mg/mL) was mixed with 1/10 volume of **9I** (3 mM final concentration). The protein/dipeptide or protein/inhibitor mixtures were combined with an equal volume of the reservoir solution containing 33% (v/v) pentaerythritol propoxylate PO/OH 5/4 (Hampton Research), 0.5 % (w/v) PEG 3350, and 100 mM Tris-HCl, pH 8.0. Crystals were grown from 2- μ L droplets by the hanging-drop vapor-diffusion method at 293 K. For each complex, the diffraction intensities were collected from a single crystal at 100 K using synchrotron radiation ($\lambda = 1.00$ Å) at the SER-CAT beamline 22-ID at the Advanced Photon Source (Argonne, USA) equipped with a MAR225 CCD detector. The datasets were indexed, integrated and scaled using the HKL software package (Table S1).³⁸

Structure Determination and Refinement

Structure determination of GCPII complexes presented here were carried out using difference Fourier methods with the ligand-free rhGCPII (PDB code 2OOT)³⁹ as a starting model. Calculations were performed with the program Refmac 5.5.⁴⁰ and the refinement protocol was interspersed with manual corrections to the model employing the program Coot.⁴¹ The restraints library and the coordinate files for individual inhibitors were prepared using the PRODRG server⁴² and the inhibitors/substrates were fitted into the positive electron density map in the final stages of the refinement. Approximately 1% of the randomly selected reflections were kept aside for cross-validation (R_{free}) during the refinement process. The quality of the final models was evaluated using the MOLPROBITY.⁴³ The data collection and refinement statistics are summarized in 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 3SJX [GCPII(E424A)/**NAAM**], 3SJG [GCPII(E424A)/**7S**], 3SJE [GCPII(E424A)/**8S**], and 3SJF (GCPII/**9I**).

Substrate Synthesis

Glycine, (*S*)-alanine, (*S*)-norleucine, (*S*)-methionine, (*S*)-aminobutyric acid, (*S*)-glutamic acid, and (*S*)-norvaline were purchased from Sigma. (*S,R*)-forms of 2-amino heptanoic, octanoic, and nonanoic acid were purchased from Fluka. N-acetylated dipeptides were synthesized using standard Fmoc-based approach on a Wang hydroxymethylphenyl-functionalized resin.⁴⁴ The identity and purity of the peptides was checked by mass

spectrometry, reversed-phase HPLC, and amino acid analysis and was determined to be > 95% (data not shown).

Determination of Kinetic Constants

Kinetic constants of N-terminally acetylated dipeptidic substrates were determined by high-performance liquid chromatography with fluorimetric detection (excitation 250 nm, emission 395 nm) of pre-column derivatized amino acids. Typically, 1-2 µg/ml of rhGCP II in 20 mM MOPS, 20 mM NaCl, pH 7.4, was reacted with 5 to 300 µM of the relevant substrate for 30 min at 37 °C in a final volume of 120 µl. The reaction was stopped by the addition of 20 µl of 100 mM EDTA, pH 9.2, and the pH of the reaction mixture was adjusted by the addition of 40 µl of 100 mM borate buffer, pH 9.0. The released amino acids were derivatized using 20 µl of 2.5 mM AccQ-Fluor reagent (Waters, Milford, USA) dissolved in acetonitrile. 30 µl of the resulting mixture was applied to a Luna C18(2)-column (250 × 4.6 mm, 5 µm particle size, Phenomenex) mounted to a Waters Alliance 2795 system equipped with a Waters 2475 fluorescence detector.

Synthesis of GCP II Inhibitors

Some of the tested inhibitors (**1I**, **4I**, **DCIBzL** & **9I**) were already reported previously by our laboratory. The other GCP II inhibitors (**2I**, **3I**, **5I-8I**) were prepared by the general procedure as follows.

To N^ε-Boc-L-lysine t-butyl ester hydrochloric acid (339 mg, 1 mmol) in 20 mL of CH₂Cl₂ at -78 °C was added triphosgene (98 mg, 0.33 mmol) followed by triethylamine (1 mL). The reaction mixture was stirred at -78 °C for 1 hr and the dry ice/acetone bath was removed. The stirring was continued for another 30 min at room temperature and the reaction mixture cooled back to -78 °C. To the reaction mixture were added the individual amino acid (1 mmol) in anhydrous DMF (10 mL) and triethylamine (1 mL). After stirring overnight, the excess solvent was removed under reduced pressure and the residue was purified by reverse phase HPLC to give urea compounds (20-50% yield). The urea compounds (0.1 mmol) were dissolved in 1 mL of TFA and stirred at room temperature for 60 min. The completion of the deprotection reaction was monitored by ESI-MS. Excess TFA was removed by reduced pressure. The residue was dissolved in DMF (5 mL) and triethylamine (0.5 mL), followed by the addition of 4-iodosuccinimide benzoate (0.12 mmol). The reaction mixture was stirred at room temperature for 3 hr. The excess solvent was removed and the residue was purified by reverse phase HPLC to give the target compounds (40-60% yield). The final purity of inhibitors was >95% as determined by analytical HPLC.

(S)-2-(3-((S)-1-carboxyethyl)ureido)-6-(4-iodobenzamido)hexanoic acid (**2I**)—

HPLC condition: H₂O/CH₃CN (0 min: 90/10 → 30 min: 50/50, 0.1% TFA), flow rate: 3 mL/min, retention time: 23 min. ¹H NMR (400 MHz, H₂O-d₂) δ: 8.17 (d, J=8.0 Hz, 2H), 7.80 (d, J=7.2 Hz, 2H), 4.40-4.44 (m, 2H), 3.41-3.44 (m, 2H), 1.95-2.05 (m, 2H), 1.86-1.89 (m, 2H), 1.67-1.72 (m, 2H), [M+H] calculated for C₁₇H₂₂IN₃O₆ 492.1 found: 491.9. Yield: 21%.

(S)-2-(3-((S)-1-carboxypropyl)ureido)-6-(4-iodobenzamido)hexanoic acid (**3I**)—

HPLC condition: H₂O/CH₃CN (0 min: 75/25 → 30 min: 40/60, 0.1% TFA), flow rate: 3 mL/min, retention time: 13 min. ¹H NMR (400 MHz, CD₃CN) δ: 7.81 (d, J=6.8 Hz, 2H), 7.46 (d, J=6.8 Hz, 2H) 4.08 (m, 1H), 4.01 (m, 1H), 3.26 (d, J=6.8 Hz, 2H), 1.70-1.75 (m, 2H), 1.63-1.68 (m, 2H), 1.60-1.64 (m, 2H) 1.32-1.38 (m, 2H), 0.84 (t, J=7.2 Hz, 3H) [M+H] calculated for C₁₈H₂₄IN₃O₆ 506.1 found: 505.8. Yield: 19%.

(S)-2-(3-((S)-1-carboxypentyl)ureido)-6-(4-iodobenzamido)hexanoic acid (5l)—

HPLC condition: H₂O/CH₃CN (0 min: 75/25 → 30 min: 40/60, 0.1% TFA), flow rate: 3 mL/min, retention time: 15 min. ¹H NMR (400MHz, MeOH-d₄) δ: 7.82 (d, J= 7.2Hz, 2H), 7.56 (d, J= 7.2 Hz, 2H), 4.25 (m, 1H), 4.22 (m, 1H), 3.31-3.39 (m, 2H), 1.83-1.89 (m, 2H), 1.62-1.67 (m, 4H), 1.45-1.49 (m, 2H), 1.32-1.39 (m, 4H), 0.89 (t, 3H) [M+H] calculated for C₂₀H₂₈IN₃O₆ 534.1 found: 533.8. Yield: 17%.

(S)-2-(3-((S)-1-carboxyhexyl)ureido)-6-(4-iodobenzamido)hexanoic acid (6l)—

HPLC condition: H₂O/CH₃CN (58/42, 0.1% TFA), flow rate: 3 mL/min, retention time: 13.5 min. ¹H NMR (400MHz, CD₃CN) δ: 7.80 (d, J= 7.2Hz, 2H), 7.49 (d, J=7.2 Hz, 2H), 4.13-4.18 (m, 1H), 4.08-4.12 (m, 1H), 3.27 (t, J= 6.8 Hz, 2H), 1.86-1.89 (m, 1H), 1.74-1.78 (m, 2H), 1.62-1.66 (m, 3H), 1.52-1.55 (m, 2H), 1.29-1.37 (m, 6H), 0.87 (t, J= 6.2 Hz, 3H) [M+H] calculated for C₂₁H₃₀IN₃O₆ 548.2 found: 547.8. Yield: 18%.

(S)-2-(3-((S)-1-carboxyheptyl)ureido)-6-(4-iodobenzamido)hexanoic acid (7l)—

HPLC condition: H₂O/CH₃CN (58/42, 0.1% TFA), flow rate: 3 mL/min, retention time: 16.5 min. ¹H NMR (400MHz, CD₃CN) δ: 7.82 (d, J= 7.2Hz, 2H), 7.50 (d, J=7.2 Hz, 2H), 4.15-4.18 (m, 1H), 4.09-4.12 (m, 1H), 3.30 (t, J= 6.8 Hz, 2H), 1.83-1.89 (m, 1H), 1.62-1.67 (m, 3H), 1.45-1.49 (m, 2H), 1.26-1.38 (m, 10H), 0.89 (t, J= 6.2 Hz, 3H) [M+H] calculated for C₂₂H₃₂IN₃O₆ 562.1 found: 561.9. Yield: 16%.

(S)-2-(3-((S)-1-carboxyoctyl)ureido)-6-(4-iodobenzamido)hexanoic acid (8l)—

HPLC condition: H₂O/CH₃CN (58/42, 0.1% TFA), flow rate: 3 mL/min, retention time: 19.5 min. ¹H NMR (400MHz, CD₃CN) δ: 7.79 (d, J= 8.0 Hz, 2H), 7.48 (d, J=8.0 Hz, 2H), 4.11-4.16 (m, 1H), 4.04-4.08 (m, 1H), 3.26 (t, J= 6.8 Hz, 2H), 1.73-1.81 (m, 1H), 1.52-1.63 (m, 3H), 1.32-1.38 (m, 2H), 1.17-1.30 (m, 12H), 0.88 (t, J= 6.2 Hz, 3H) [M+H] calculated for C₂₃H₃₄IN₃O₆ 576.1 found: 575.8. Yield: 14%.

In vitro GCPII Inhibitory Constants

Inhibition constants were determined using a fluorescence-based assay according to a previously reported procedure.⁴⁵ Briefly, lysates of LNCaP cell extracts (25 μL) were incubated with the inhibitor (12.5 μL) in the presence of 4 μM NAAG (12.5 μL) for 120 min. The amount of the released glutamate was determined by incubating with a working solution (50 μL) of the Amplex Red Glutamic Acid Kit (Invitrogen Corp., CA, USA) for 60 min. The fluorescence was measured using a VICTOR³V multilabel plate reader (Perkin Elmer Inc., Waltham, MA, USA) with excitation at 490 nm and emission at 640 nm. Inhibition curves were determined using semi-log plots and IC₅₀ values were calculated. Assays were performed in triplicate. Enzyme inhibitory constants (*K_i* values) were generated using the Cheng-Prusoff conversion. Data analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California).

Quantum Chemical Calculations and QM/MM Modeling

All QM/MM calculations were carried out with the COMQUM program.⁴⁶ In the current version, the program uses Turbomole 5.7⁴⁷ for the QM part and AMBER 8 (University of California, San Francisco, U. S. A.) with the Cornell force field⁴⁸ for the MM part. The details of the QM/MM procedure that are essentially identical to the ones described in Ref.²³ can be found in the Supplementary Material.

The Protein (GCPII) Setup

All structural models used in QM/MM calculations were based on the reported crystal structures and on our previous work²³ where we carefully described the strategy to include missing loops, addition of hydrogens, protonation states of His residues, and construction of

solvation sphere, including initial simulated annealing protocol leading to the optimization of positions of all the atoms missing in the crystal structures. All these structural details can be found in the PDB files deposited as the Supplementary Material. Therefore, starting from the QM/MM optimized structure of the NAAG (GCPII substrate) complexed with the enzyme,²³ we have replaced NAAG with the studied substrates/inhibitors (**NAAM**, **7S**, **8S**, **S8**, **I8**, **I10**) using their experimental crystal structures as templates. In case of **8I**, for which the crystal structure has not been yet determined, we used GCPII/DCIBzL and GCPII(E424A)/**7S** structures as templates for positioning its P1 and P1' parts, respectively. Due to the high similarity of the structures, we considered this approach as physically more sound, since the (highly-optimized) QM/MM structure already contained all hydrogen atoms and missing parts of the protein. This approach is also justified a posteriori by an excellent agreement of the equilibrium QM/MM and crystal structures for a given enzyme/inhibitor complex. The only significant structural change involved the Lys699 residue which swings by ~3.5 Å upon binding of the inhibitors with longer alkyl chains as described above. Also, we have tried to carefully adapt the positions of 2-3 water molecules in the vicinity of the active site to match their positions in the crystal structures. The quantum region in the calculations consisted of the following residues: two Zn²⁺ ions, bringing hydroxide moiety, inhibitor, side chains of Phe209, Arg210, Asn257, His377, Asp387, Glu424, Glu425, Asp453, Arg463, Asp465, Arg534, Arg536, Tyr552, His553, Lys699, Tyr700, the Gly427-Leu428 chain (capped by CHO moiety of the Phe426 at the N-terminus and NH-CH₃ moiety of the Leu429 residue at the C-terminus), the Ser517-Asp520 chain (capped by CHO moiety of the Gly516 at the N-terminus and NH-CH₃ moiety of the Phe521), and 6-8 water molecules in the vicinity of the active site. It resulted in fairly large QM system of ~350 atoms.

Quantum Chemical Calculations

All quantum chemical calculations were performed at the density functional theory (DFT) level. Geometry optimizations were carried out at the Perdew-Burke-Ernzerhof (PBE) level.⁴⁹ The DFT/PBE calculations were expedited by expanding the Coulomb integrals in an auxiliary basis set - the resolution-of-identity (RI-J) approximation.⁵⁰ In QM/MM optimizations, the def2-SVP basis set was employed for all atoms.⁵¹ The calculations of the interaction energy of the non-polar side chains of the inhibitors with the S1' residues and its decomposition into the pair contributions was carried out using the recent DFT-D3 computational protocol (DFT with the empirical dispersion) of Grimme⁵² that was shown to yield, in combination with the B-LYP functional⁵³ and TZVPP basis set⁵¹ excellent values of the interaction energies for non-covalently bound complexes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

rhGCPH	recombinant human glutamate carboxypeptidase II
QM/MM	quantum mechanics/molecular mechanics
NAAM	N-acetyl-aspartyl-methionine
NAAG	N-acetyl-aspartyl-glutamate
ANO	2-aminononanoic acid
AOC	2-aminooctanoic acid
SAR	structure-activity relationship
ESI-MS	electrospray ionization mass spectroscopy
PET	positron emission tomography
Ac	acetyl group
GCPH	glutamate carboxypeptidase II
SPECT-CT	single photon emission computed tomography
HPLC	high performance liquid chromatography
DFT	density functional theory
PBE level	Perdew-Burke-Ernzerhof level
PCa	prostate cancer

Figure 1.

Panel A Superposition of NAAM, NAAO and NAAN substrates in the substrate binding pocket of GCPII(E424A). The substrates are shown in stick representation with carbon atoms colored green (NAAM), gray (NAAO), and magenta (NAAN). Selected GCPII residues surrounding the binding pocket are shown in line representation. The zinc ions are shown as orange spheres.

Panel B Superposition of NAAG (PDB code 3BXM) and NAAN substrate binding sites of GCPII. Substrates and the amino acids Lys699 are shown in stick representation, zinc ions and waters as orange and red spheres, respectively. Selected GCPII residues surrounding the binding pocket are shown in line representation. Atoms are colored as follows: cyan (NAAG carbon), green (NAAN carbon), blue (nitrogen), and red (oxygen). Notice the displacement of the Lys699 side chain by 3.9 Å in the NAAN complex (green) due to steric clash with the NAAN side chain.

Panel C and D The S1' pocket plasticity defined by the "Lys699 swing".

Panel C the composite figure showing GCPII substrate binding site (surface representation) derived from the NAAG (PDB code 3BXM) complex and NAAN (stick representation). Notice the steric clash between the side chain of Lys699 (green) and the side chain of the C-terminal residue in NAAN.

Panel D GCPII substrate binding site (surface representation) from the complex with NAAN (stick representation). The swing-out of the Lys699 side chain (green) enlarges the S1' pocket to accommodate longer (or more bulky) side chains of P1' part of substrates/inhibitors. The Figure was generated using The PyMOL Molecular Graphics System, Version 0.99, Schrödinger, LLC.

Figure 2.

The stereo view of the electron density map of the GCPII/**9I** complex. The $2F_o-F_c$ map is contoured at 1σ (blue) and the F_o-F_c electron density maps contoured at -3σ (red) and $+3\sigma$ (green). Carbon atoms of the inhibitor and GCPII are colored brown and gray, respectively. The following coloring scheme was used for individual atoms: oxygen (red), nitrogen (blue), iodine (green), sulphur (yellow), zinc (pink).

Figure 3.

A stereo view of superimposed active site regions of GCPII/**9I** and GCPII(E424A)/**NAAM** complexes (inhibitor and its parent substrate). A fragment of the GCPII/**9I** (*wild-type GCPII and 9I inhibitor*) complex is shown in light-brown and an equivalent fragment of the GCPII(E424A)/**NAAM** (*the inactive mutant and NAAM substrate*) complex is shown in green. The following coloring scheme was used for individual atoms: oxygen (red), nitrogen (blue), iodine (green), sulfur (yellow). Two active site zinc atoms are shown as pink spheres and the substrate/inhibitor are shown in a thick ball-and-stick representation.

Figure 4.

A model system used for the calculations of interaction energies, the S1' residues (side chains of Phe209, Asn257, Leu428, backbone of Gly427 and Gly518, and non-polar part of the Lys699 side chain) interacting with the non-polar side chains of the substrates/inhibitors. The model system truncated from the GCPII(E424A)/NAAM complex (Met side chain and model functional groups representing the key interacting residues from the enzyme) is used in the figure.

Table 1

Formulas and kinetic parameters of novel GCPII dipeptidic substrates. Ac-Asp-Glu (**NAAG**) – natural GCPII substrate in mammalian nervous system; Ac-Asp-Met (**NAAM**) – non-natural GCPII substrate from the dipeptidic library screen. **1S** – **8S** – novel GCPII dipeptidic substrates featuring non-polar aliphatic side chain at the P1' position. The kinetic parameters were determined by saturation kinetics employing precolumn derivatization of the reaction products (*i.e.* released C-terminal amino acids) with AccQ-Fluor, followed by HPLC separation on a C18(2) Luna column and fluorimetric detection.

	K_M [μM]	k_{cat} [s^{-1}]	k_{cat} / K_M [$\text{L}\cdot\text{mmol}^{-1}\cdot\text{s}^{-1}$]
1S (Ac-Asp-Gly)	NA [*]	NA	NA
2S (Ac-Asp-Ala)	85 ± 21	0.23 ± 0.05	2.7
3S (Ac-Asp-Val)	29 ± 8	0.25 ± 0.03	8.6
4S (Ac-Asp-nVal)	31 ± 12	0.42 ± 0.05	13.5
5S (Ac-Asp-nIle)	19 ± 4	0.24 ± 0.01	12.6
6S (Ac-Asp-Ile)	12 ± 1	0.44 ± 0.04	36.7
7S (Ac-Asp-Ala)	8 ± 2	0.60 ± 0.03	75.0
8S (Ac-Asp-Ala)	10 ± 2	0.62 ± 0.10	62.0
NAAM	$24.8 \pm 2.9^{\text{@}}$	0.07 ± 0.002	2.9
NAAG	$1.2 \pm 0.5^{\text{\#}}$	$1.1 \pm 0.2^{\text{\#}}$	930 [#]

* not analyzed, substrate hydrolysis below the detection limit

[@] from ref.²¹

[#] from ref.²⁹

Table 2

Effects of the K699S mutation on **NAAG**, **NAAM**, and **8S** hydrolysis. The kinetic parameters of wild-type (WT) and mutated (K699S) GCPII towards individual substrates were determined by saturation kinetics. The reaction products were derivatized with AccQ-Fluor and separated by HPLC on a C18(2) Luna column with fluorimetric detection.

Substrate	K_M [μM]	k_{cat} [s^{-1}]		k_{cat}/K_M [$\text{L}\cdot\text{mmol}^{-1}\cdot\text{s}^{-1}$]	
		K699S	WT	K699S	WT
NAAG	$1.2 \pm 0.5^*$	40.5 ± 22.7	$1.1 \pm 0.2^*$	0.34 ± 0.08	930^*
NAAM	7.5 ± 1.6	2.6 ± 1.1	0.32 ± 0.07	0.25 ± 0.04	42.7
8S	10.4 ± 2.5	2.9 ± 0.7	0.62 ± 0.10	0.37 ± 0.05	59.6
					127.6

* The kinetic parameters of the **NAAG** hydrolysis by rhGCPII were taken from ref.²⁹ and are included herein for the purpose of comparison. These values were determined by the radioenzymatic assay using ^3H -NAAG as a substrate.

Table 3

Inhibition of GCPII by novel substrate-based inhibitors
Inhibitory properties of the novel compounds were determined using the Amplex Red assay and the results are summarized below.

General inhibitor template					
Inhibitor	X moiety	Ki (nM)	CI (95% confidence)	M.W	ClogD
ZJ-43	ZJ-43	1.08 (0.75 [*])	0.85-1.36	304.13	-6.13
1I	X = -H	4390 (1320 [*])	3344-5762	477.04	-3.76
2I	X = -CH ₃	325	231-458	491.06	-3.41
3I	X = -CH ₂ CH ₃	121	86-165	505.07	-2.88
4I	X = -(CH ₂) ₂ CH ₃	145 (52.3 [*])	96-219	519.09	-2.35
5I	X = -(CH ₂) ₃ CH ₃	56	34-92	533.10	-1.82
6I	X = -(CH ₂) ₄ CH ₃	20	10-40	547.12	-1.29
7I	X = -(CH ₂) ₅ CH ₃	72	43-123	561.13	-0.76
8I	X = -(CH ₂) ₆ CH ₃	29	21-38	575.15	-0.23
9I	X = -Met	23	12-44	551.40	-2.37
10I (DCBzL)	X = -Glu	0.01 [*]	0.007 – 0.013	549.06	-5.16

^{*} values from ref.³²

Table 4

Calculated interaction energies of the model ligands representing S1' residues with the P1' side chains of the selected substrates/inhibitors using B-LYP(+D)/TZVPP method and QM/MM optimized geometries of GCPII/ligand complexes. All values are in kcal.mol⁻¹.

	Met	AOC	ANO
Phe209	-1.4	-2.4	-2.2
Asn257	-4.8	-1.7	-2.4
Gly427(bb)	-1.3	-1.9	-2.1
Leu428	-1.3	-2	-2.1
Gly518(bb)	-2.1	-2	-1.4
Lys699(n-p)	-0.3	-1.4	-1.6
ΣE_{ij}	-11.2	-11.4	-11.5
$E_{int}(\text{total})$	-11.1	-11.3	-11.5