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Dipeptide inhibitors of the prostate specific membrane antigen (PSMA): A comparison of urea and thiourea derivatives

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ABSTRACT

Glutamate carboxypeptidase II (GCP(II)), also known as the prostate-specific membrane antigen (PSMA), is a transmembrane zinc(II) metalloenzyme overexpressed in prostate cancer. Inhibitors of this receptor are used to target molecular imaging agents and molecular radiotherapy agents to prostate cancer and if the affinity of inhibitors for GCP(II)/PSMA could be improved, targeting might also improve. Compounds containing the dipeptide OH-Lys-C(O)-Glu-OH (compound 3), incorporating a urea motif, have high affinity for GCP(II)/PSMA. We hypothesized that substituting the zinc-coordinating urea group for a thiourea group, thus incorporating a sulfur atom, could facilitate stronger binding to zinc(II) within the active site, and thus improve affinity for GCP (II)/PSMA. A structurally analogous urea and thiourea pair (HO-Glu-C(O)-Glu-OH - compound 5 and HO-Glu-C (S)-Glu-OH - compound 6) were synthesized and the inhibitory concentration (IC_{50}) of each compound measured with a cell-based assay, allowing us to refute the hypothesis: the thiourea analogue showed 100-fold weaker binding to PSMA than the urea analogue.

In the last 5 years, clinical management of prostate cancer has been transformed by the introduction of radiopharmaceuticals targeting glutamate carboxypeptidase II (GCP(II)), also known as the prostate-specific membrane antigen (PSMA).[1–4] Positron emission tomography/computed tomography (PET/CT) scans of prostate cancer patients imaged with radiopharmaceuticals that target this receptor, such as [^{68}Ga]Ga-HBED-CC-PSMA[5] and [^{68}Ga]Ga-THP-PSMA,[6] can provide clinically useful information about the location and spread of disease.[1–4,7,8] PET/CT scans allow clinicians to accurately stage patients and alter treatment plans accordingly.[1,7] Molecular radiotherapy with [^{177}Lu]Lu-PSMA-617 is currently being evaluated in a multinational phase 3 trial[9] (NCT NCT03511664). As a consequence, the demand for PSMA imaging agents is growing year on year. The excellent performance of these imaging agents is underpinned by two factors: i) GCP(II)/PSMA is very specific to prostate cancer, with 100–1000 fold higher expression in prostate cancer compared to normal prostate and low endogenous expression in other organs[10,11]; ii) radiopharmaceuticals are designed to be very specific to GCP(II)/PSMA with K_i (equilibrium constant) and IC_{50} (half maximal inhibitory concentrations) values in the low nM to sub-nM range.[5,6,12,13]

GCP(II)/PSMA is a transmembrane zinc(II) metalloenzyme that catalyzes the cleavage of terminal glutamates.[14] Its active site is specific for C-terminal glutamate residues, binding them tightly. A feature of the active site is the presence of two zinc(II) ions which participate in catalyzing the cleavage of the peptide bond between the terminal glutamate and the remainder of the substrate.[15,16] The natural substrates of GCP(II)/PSMA - *N*-Acetyl-L-aspartyl-L-glutamate (NAAG) and poly-glutamated-folates[14] - are shown in figure 1 (compounds 1 & 2, respectively). Examples of the dipeptide urea-based targeting motifs used in the majority of GCP(II)/PSMA targeted radiopharmaceuticals[5,6,11,17,18] are also shown in figure 1. The OH-Lys-C(O)-Glu-OH (compound 3) and OH-Cys-C(O)-Glu-OH (compound 4) motifs were developed by rational design from the natural substrates of GCP(II)/PSMA[19] and are remarkably small, simple and potent.[5,12,17–19] These

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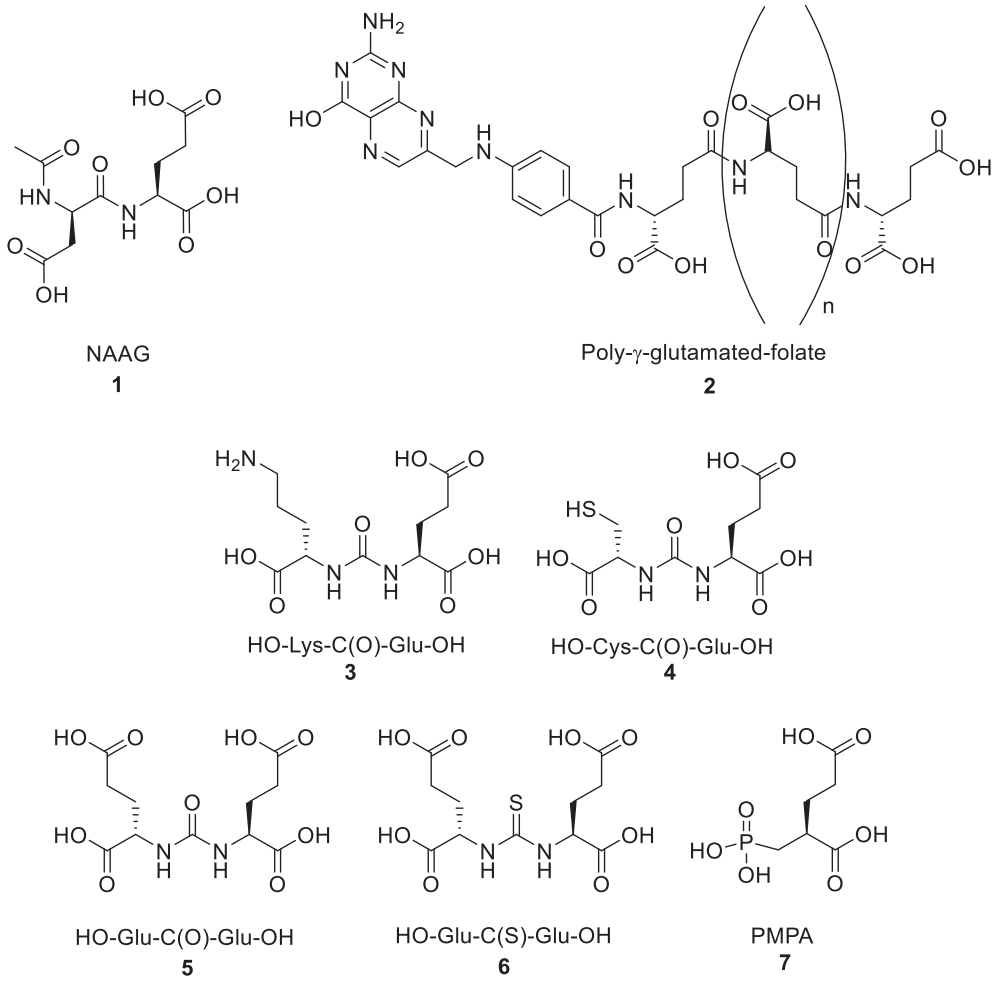

Fig. 1. Structures of the natural substrates of GCP(II)/PSMA (top row) and known inhibitors and the novel inhibitor (compound 6 - HO-Glu-C(S)-Glu-OH) addressed in this communication.

ligands preserve the structure of the terminal glutamate but replace the peptide bond with a urea bond, thus reducing the electrophilicity of the carbonyl carbon atom and providing stability against enzymatic cleavage by GCP(II)/PSMA. The urea functional group links the glutamate to a second amino acid (L-lysine or L-cysteine), which can be used to functionalize this inhibitory motif.[15] For example, it can be converted into a PET radiotracer by adding a prosthetic group containing covalently-bound radionuclide such as fluorine-18,[20,21] or a chelator allowing radiometals, such as gallium-68, to be incorporated.[5,6,18,22]

Despite the success of the dipeptide urea-based motif and its proven utility in the clinic, it is expected that further improvement in affinity for GCP(II)/PSMA is possible. Valuable information to guide rational design is now available from X-ray crystallography studies,[23–25] including evidence that the urea oxygen coordinates to Zn(II) in the active site,[26] and many structural modifications to improve affinity have been attempted previously.[12,16] The main conclusions from this body of published work are: (i) the conservation of the terminal glutamate is extremely important and any modifications to it reduce affinity[12,16]; (ii) the ability of an inhibitor to bind the zinc(II) atoms in the active site also determines affinity.[16] Urea groups,[19,25,26] phosphonates,[13,25] phosphinates[13,25] and phosphonamidates[25,27] have been shown to bind strongly to zinc within GCP(II)/PSMA, with the urea closely mimicking the structure of a peptide bond and the others mimicking the tetrahedral transition state/intermediate (with an sp^3 hybridized carbon) during peptide bond cleavage.[15] This shows that the zinc(II)-binding group is amenable to variation and provides an opportunity to improve affinity (by enhancing Zn-binding) through modification of this group. It is also important that the selected zinc-binding group is resistant to enzymatic cleavage by GCP(II)/PSMA and that it can link the terminal glutamate to the rest of the inhibitor, which is used for functionalization.

The presence of zinc(II) ions in the active site has previously prompted investigators to look to thiols as zinc-binding motifs in GCP (II)/PSMA inhibitors, with limited success.[28] However, to date thiourea-based inhibitors have not been tested as GCP(II)/PSMA ligands. The rationale for replacing oxygen with sulfur in an inhibitor for a zinc(II)-based metalloenzyme is twofold. First, zinc(II) ions and sulfur-based ligands, including thiourea, have a strong affinity for each other and typically form highly stable complexes.[29,30] Zinc(II)-sulfur interactions are ubiquitous in biology, including functional processes (for example heat shock protein Hsp33 in which zinc(II) binds to redox-active thiolate groups that induce a conformation change upon oxidation[31]), and stabilization of structures (for example zinc(II) finger motifs that are common to many proteins[32]). Second, the resonance within the thiourea group favours more negative charge on the sulfur compared to the oxygen of the urea group; this would be expected to allow stronger interaction with the zinc(II) ions. We therefore hypothesized that replacing the urea with a thiourea in this class of dipeptide inhibitors could improve affinity.

To test this hypothesis, we elected to modify the symmetrical urea-based inhibitor compound 5 (HO-Glu-C(O)-Glu-OH), which is reported to have a $K_i = 8$ nM for GCP(II)/PSMA.[12] The thiourea analog compound 6 (HO-Glu-C(S)-Glu-OH), was designed to conserve the interactions in the glutamate binding pocket and remain resistant to enzymatic cleavage, but to have enhanced interactions with the zinc(II) ions through the

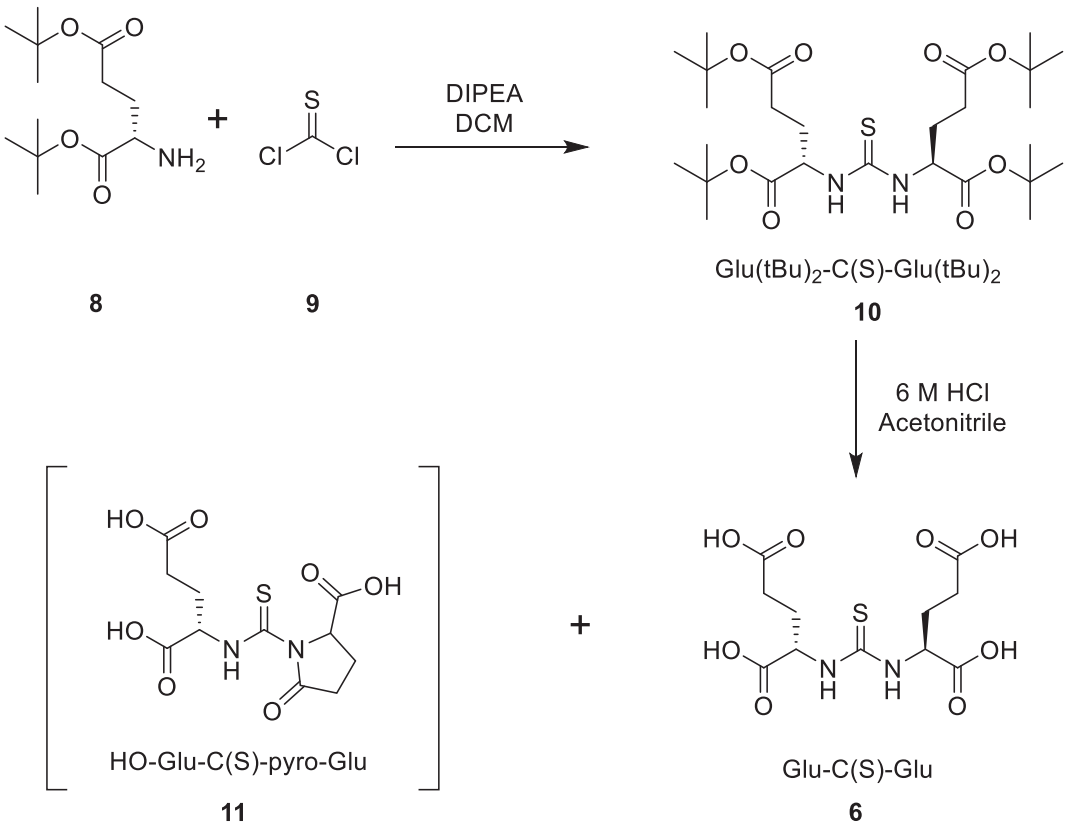

Fig. 2. Synthesis route for the production of compound 6 (HO-Glu-C(S)-Glu-OH) and cyclized side product compound 11 (HO-Glu-C(S)-pyroGlu).

presence of the sulfur atom. The formation of thiourea is synthetically achievable through an isothiocyanate intermediate, using existing well-characterized chemistry.[33]

Compounds 5 and 6 were synthesized by similar two-step synthetic routes. The reaction schemes for compound 5 are found in the supplemental files and the reaction scheme for compound 6 is found in figure 2. The first step was the formation of a urea or thiourea linkage between two *t*-butyl-protected L-glutamate residues (compound 8). For the urea compound 14 ($Glu(tBu)_2$-C(O)-$Glu(tBu)_2$), triphosgene was used to generate an isocyanate intermediate, followed by reaction with another equivalent of compound 8, to yield the desired product. For the thiourea compound 10 ($Glu(tBu)_2$-C(S)-$Glu(tBu)_2$), thiophosgene was used to generate an isothiocyanate intermediate with subsequent formation of a thiourea bond.

The second synthetic step removed the *t*-butyl protecting groups before purification of the final compounds. Compound 14 was deprotected using trifluoroacetic acid in the presence of phenol and triisopropylsilane as scavengers, and then isolated using semi-preparative reverse phase HPLC purification. However, when compound 10 was similarly deprotected using trifluoroacetic acid/ phenol/ triisopropylsilane, the major species obtained was compound 11 which contained a γ-lactam pyroglutamic acid residue. Similar cyclization reactions are well-known[34] and many peptides and proteins naturally have a pyroglutamic acid at their N terminus.[35] This suggests that compound 10 is more prone than compound 14 to dehydration under these conditions. This is likely to be due to the stronger preference of thiourea for the enol/enolate resonance form, due to sulfur's weaker π-bonding and its concomitant ability to stabilize the negative charge.

As cyclisation was particularly prevalent in high acid, low water conditions, the reaction conditions were modified to avoid it: compound 10 was reacted in a 2:1 solution of 6 M HCl and acetonitrile for 8 h, followed by neutralization and purification by semi-preparative HPLC. This increased yields of compound 6. Compounds 5 and 6 were

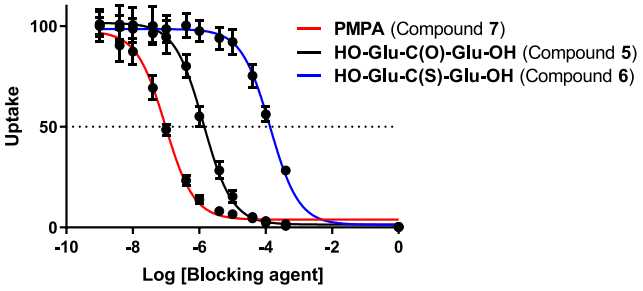

Fig. 3. Inhibition curves for compounds 5, 6 and 7, (HO-Glu-C(O)-Glu-OH, HO-Glu-C(S)-Glu-OH and PMPA) with [^{67}Ga]Ga-DOTA-PSMA(617) at 1 nM as the probe. Values are averaged across triplicate assays. For each assay n = 4 wells at each concentration. Note that non-specific binding of [^{67}Ga]Ga-DOTA-PSMA (617) with non-GCP(II)PSMA-expressing cells (DU145) was used as the nominal value at 1 M inhibitor.

characterized by nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (supplemental files).

To ensure that compound 6 was resistant to cyclisation under conditions required for *in vitro* affinity measurements, appropriate NMR studies were conducted. Aqueous solutions of compound 5 and compound 6 at pH 7 (pH adjusted with phosphate-buffered saline (PBS) and ammonium acetate) were monitored using ^{1}H NMR (400 MHz) for 48 h. Both inhibitors were stable, with no cyclisation detected under these conditions. ^{1}H NMR (400 MHz) was also used to monitor the stability of compound 6 with respect to cyclisation in the presence of GCP(II)/ PSMA-expressing cells (DU145-PSMA); the thiourea ligand was found to be stable in these conditions.

Inhibition assays (IC_{50} assays) were conducted in triplicate to compare compound 7 (2-(phosphonomethyl)pentanedioic acid, (PMPA)), compound 5 and compound 6 over a concentration range of 1

Table 1
Literature K_i values [12,13] and experimental IC_{50} values for the inhibitors and the relative K_i ratios and relative IC_{50} ratios used to compare the affinity of the inhibitors.

	PMPA Compound 7	HO-Glu-C(O)-Glu-OH Compound 5	HO-Glu-C(S)-Glu-OH Compound 6
K_i (literature values)	0.3 nM [13]	8 nM [12]	–
IC_{50} (experimentalvalues)	94 ± 4 nM	1340 ± 70 nM	135000 ± 6600 nM
Relative K_i ratio	Compound 5 K_i / Compound 7 K_i		Ratio 26.7
Relative IC_{50} ratio	Compound 5 IC_{50} / Compound 7 IC_{50}		Ratio 14.3
Relative IC_{50} ratio	Compound 6 IC_{50} / Compound 7 IC_{50}		Ratio 1436
Relative IC_{50} ratio	Compound 6 IC_{50} / Compound 5 IC_{50}		Ratio 101

nM to 400 μM. Compound 7 is a widely-used GCP(II)/PSMA inhibitor that has a phosphonate zinc(II)-binding group ($K_i = 0.3$ nM[13]), and was included as an additional control. These competitive binding studies utilized GCP(II)/PSMA-expressing cells (DU145-PSMA[35]) and the radiolabeled PSMA imaging agent [^{67}Ga]Ga-DOTA-PSMA(617)[6,37] as the probe (1 nM DOTA-PSMA(617)). Non-GCP(II)/PSMA-expressing cells (DU145[36]) were used as a control to account for non-specific binding. The IC_{50} assays revealed large differences in affinity between the three inhibitors (figure 3): 1340 ± 70 nM, 135000 ± 6600 nM and 94 ± 4 nM for compounds 5, 6 and 7, respectively. Table 1 shows the relative IC_{50} ratios for the three inhibitors. K_i values from isolated enzyme assays have been previously reported for compound 7[13] and compound 5[12]. The relative K_i ratio for these two compounds (ratio 26.7) match well with the relative IC_{50} ratio determined for the same inhibitors using our cell-based assay (ratio 14.3). A summary of the relationship between IC_{50} and K_i and the conditions under which their relative ratios can be directly compared[38] is available in supplemental files.

The 100-fold increase in the IC_{50} value for the newly synthesized thiourea compared to the urea compound shows that the compound 6 is a much less potent inhibitor than compound 5, and therefore this modification worsens rather than improves affinity – the opposite of our hypothesis. The quantification and stability tests performed confirmed that compound 6 was at the required concentration during the assay (supplemental files) and that it was stable for its duration. Therefore, this value is a true reflection of the change in IC_{50} value resulting from the replacement of urea with thiourea.

The unexpected low affinity of compound 6 could be due to the longer C═S bond relative to the C═O bond (1.71 Å[39] and 1.26 Å[40] respectively). Additionally, Zn-S bonds are also typically longer than Zn-O bonds[41]. Such changes could detrimentally perturb the interactions of other key functional inhibitor groups within the active site; the additive enthalpic cost of these weakened interactions may counteract any gain in affinity caused by the stronger bond between the zinc(II) ion and the thiourea sulfur atom. This is consistent with previous findings, which suggest that the effectiveness of a zinc(II) ion binding group for GCP(II)/PSMA ligands is dependent on maintaining the glutamate interactions within the active site[16].

This work expands existing knowledge about GCP(II)/PSMA and inhibitor design. Although the thiourea modification weakened affinity for the receptor, further investigation into novel ways to improve the affinity would be extremely valuable and could impact prostate cancer management.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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