Bioorg. Med. Chem. Lett. 42 (2021) 128044

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

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

Jennifer D. Young [a], Michelle T. Ma [a], Thomas R. Eykyn [a], R. Andrew Atkinson [b], Vincenzo Abbate [c], Agostino Cilibrizzi [c], Robert C. Hider [c], Philip J. Blower [a,*]

[a] School of Biomedical Engineering and Imaging Sciences, King's College London, London, United Kingdom
[b] Centre for Biomolecular Spectroscopy and Randall Division of Cell and Molecular Biophysics, King's College London, London, United Kingdom
[c] Institute of Pharmaceutical Science, King's College London, London, United Kingdom

ARTICLE INFO

*Keywords:*
Glutamate carboxypeptidase II
GCP(II)
Prostate-specific membrane antigen
PSMA
Prostate cancer
Zinc(II) metalloenzyme
Thiourea

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

* Corresponding author at: School of Biomedical Engineering and Imaging Sciences, 4th Floor, Lambeth Wing, St Thomas' Hospital, Westminster Bridge Road, London SE17EH, United Kingdom.
*E-mail addresses:* jennifer.d.young@kcl.ac.uk (J.D. Young), michelle.ma@kcl.ac.uk (M.T. Ma), thomas.eykyn@kcl.ac.uk (T.R. Eykyn), andrew.atkinson@ipbs.fr (R.A. Atkinson), vincenzo.abbate@kcl.ac.uk (V. Abbate), agostino.cilibrizzi@kcl.ac.uk (A. Cilibrizzi), robert.hider@kcl.ac.uk (R.C. Hider), philip.blower@kcl.ac.uk (P.J. Blower).

https://doi.org/10.1016/j.bmcl.2021.128044
Received 1 February 2021; Received in revised form 8 April 2021; Accepted 11 April 2021
Available online 16 April 2021

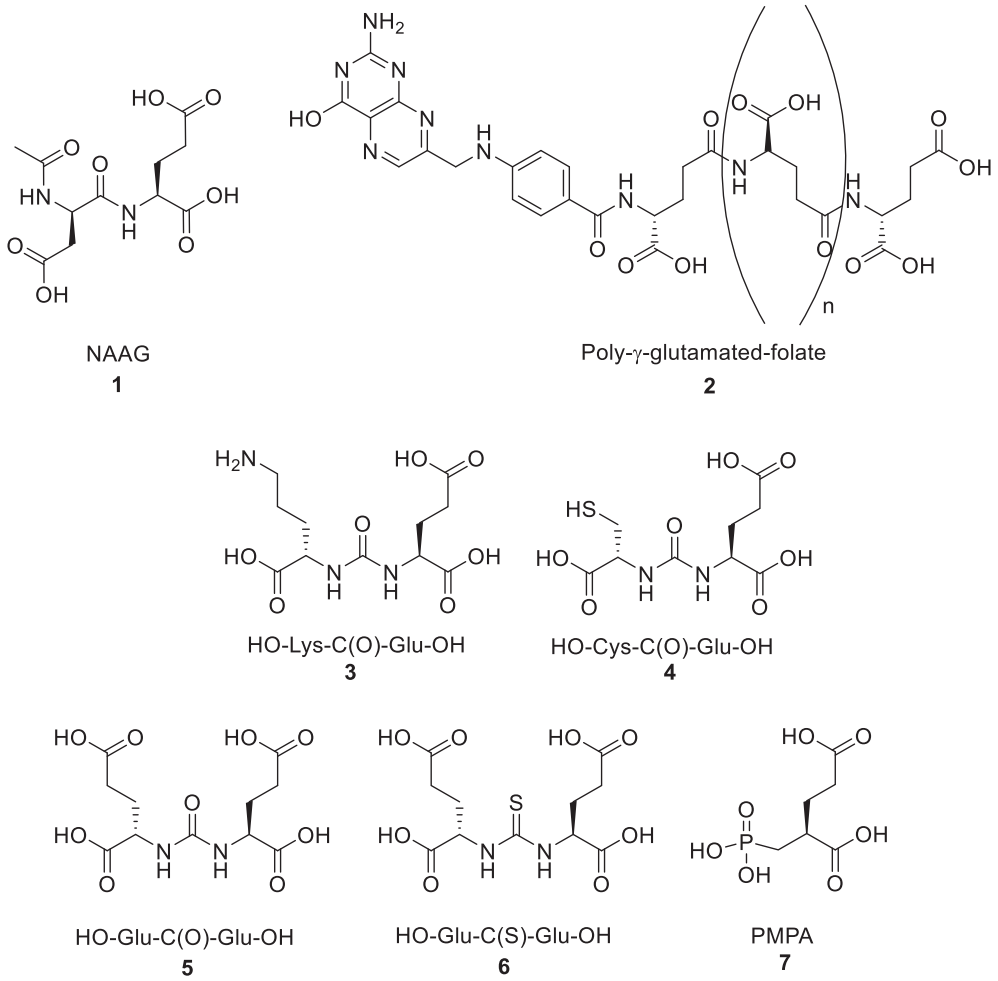


**Fig. 1.** Structures of the natural substrates of GCP(II)/PSMA (top row) and known inhibitors and the novel inhibitor (compound 6 - HO-Glu-C(S)-Glu-OH) addressed in this communication.

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

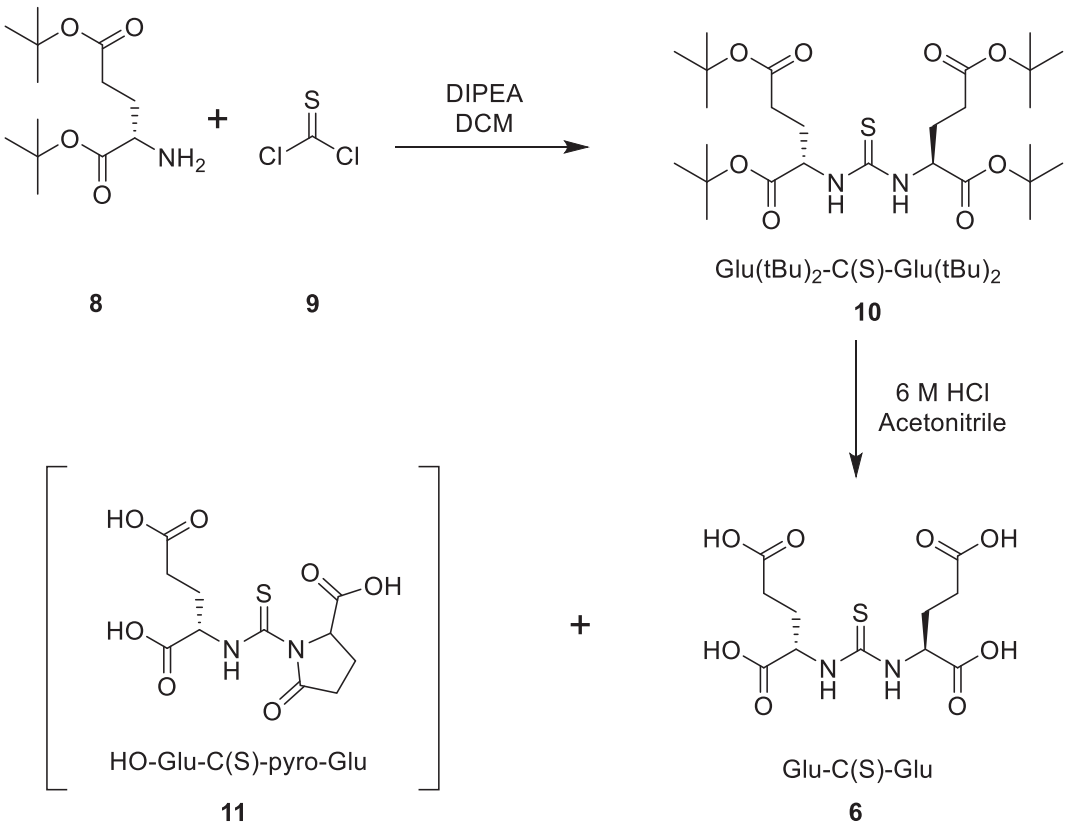


**Fig. 2.** Synthesis route for the production of compound 6 (HO-Glu-C(S)-Glu-OH) and cyclized side product compound 11 (HO-Glu-C(S)-pyroGlu).

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

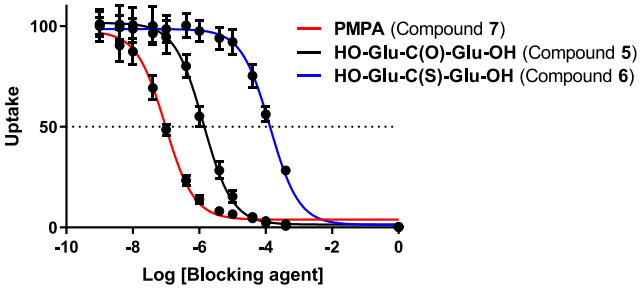


**Fig. 3.** Inhibition curves for compounds 5, 6 and 7, (HO-Glu-C(O)-Glu-OH, HO-Glu-C(S)-Glu-OH and PMPA) with [$^{67}$Ga]Ga-DOTA-PSMA(617) at 1 nM as the probe. Values are averaged across triplicate assays. For each assay n = 4 wells at each concentration. Note that non-specific binding of [$^{67}$Ga]Ga-DOTA-PSMA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

Funding: JDY was funded by the King's College London and Imperial College London EPSRC Centre for Doctoral Training in Medical Imaging (EP/L015226/1) and Theragnostics Limited. AC was funded by Theragnostics Ltd. MTM was supported by a Cancer Research UK Career Establishment Award (C63178/A24959). We acknowledge financial support from: King's Health Partners R&D Challenge Fund; MRC Confidence in concept (MC_PC_17164); the Centre of Excellence in Medical Engineering funded by the Wellcome Trust and EPSRC (WT 088641/Z/09/Z); KCL and UCL Comprehensive Cancer Imaging Centre funded by CRUK and EPSRC in association with the MRC and DoH (England); EPSRC Programme Grant ([#EP/S032789/1); and the NIHR Biomedical Research Centre award to Guy's and St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust. The views expressed are those of the authors and not necessarily those of the NHS, NIHR or DoH. The Centre for Biomolecular Spectroscopy is funded by the Wellcome Trust (202767/Z/16/Z) and British Heart Foundation (IG/16/2/32273).

## 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.

## References

1 Kulkarni M, Hughes S, Mallia A, et al. The management impact of 68gallium-tris (hydroxypyridinone) prostate-specific membrane antigen (68Ga-THP-PSMA) PET-CT imaging for high-risk and biochemically recurrent prostate cancer. *Eur J Nucl Med Mol Imaging*. 2020;47(3):674–686. https://doi.org/10.1007/s00259-019-04643-7.
2 Müller J, Ferraro DA, Muehlematter UJ, et al. Clinical impact of 68 Ga-PSMA-11 PET on patient management and outcome, including all patients referred for an increase in PSA level during the first year after its clinical introduction. *Eur J Nucl Med Mol Imaging*. 2019;46(4):889–900. https://doi.org/10.1007/s00259-018-4203-0.
3 Afaq A, Alahmed S, Chen S, et al. 68 Ga-PSMA PET/CT impact on prostate cancer management. *J Nucl Med*. 2018;59:89–92. https://doi.org/10.2967/jnumed.117.192625.
4 Roach PJ, Francis R, Emmett L, et al. The impact of 68 Ga-PSMA PET/CT on management intent in prostate cancer: results of an Australian prospective multicenter study. *J Nucl Med*. 2018;59(1):82–88. https://doi.org/10.2967/jnumed.117.197160.
5 Eder M, Schäfer M, Bauder-Wüst U, et al. 68Ga-complex lipophilicity and the targeting property of a urea-based PSMA inhibitor for PET imaging. *Bioconjug Chem*. 2012;23(4):688–697. https://doi.org/10.1021/bc200279b.
6 Young JD, Abbate V, Imberti C, et al. 68Ga-THP-PSMA: a PET imaging agent for prostate cancer offering rapid, room-temperature, 1-step kit-based radiolabeling. *J Nucl Med*. 2017;58(8):1270–1277. https://doi.org/10.2967/jnumed.117.191882.
7 Hofman MS, Eu P, Jackson P, et al. Cold kit PSMA PET imaging: phase I study of 68Ga-THP-PSMA PET/CT in patients with prostate cancer. *J Nucl Med*. 2018;59:625–631. https://doi.org/10.2967/jnumed.117.199554.
8 Hofman MS, Lawrentschuk N, Francis RJ, et al. Prostate-specific membrane antigen PET-CT in patients with high-risk prostate cancer before curative-intent surgery or radiotherapy (proPSMA): a prospective, randomised, multi-centre study. *Lancet*. 2020;6736(20):1–9. https://doi.org/10.1016/s0140-6736(20)30314-7.
9 Rahbar K, Bodei L, Morris MJ. Is the vision of radioligand therapy for prostate cancer becoming a reality? An overview of the phase III VISION trial and its importance for the future of theranostics. *J Nucl Med*. 2019;60(11):1504–1506. https://doi.org/10.2967/jnumed.119.234054.
10 Ghosh A, Heston WDW. Tumor target prostate specific membrane antigen (PSMA) and its regulation in prostate cancer. *J Cell Biochem*. 2004;91(3):528–539. https://doi.org/10.1002/jcb.10661.
11 O'Keefe DS, Bacich DJ, Heston WDW. Comparative analysis of prostate-specific membrane antigen (PSMA) versus a prostate-specific membrane antigen-like gene. *Prostate*. 2004;58:200–210. https://doi.org/10.1002/pros.10319.
12 Kozikowski AP, Zhang J, Nan F, et al. Synthesis of urea-based inhibitors as active site probes of glutamate carboxypeptidase II: efficacy as analgesic agents. *J Med Chem*. 2004;47(7):1729–1738. https://doi.org/10.1021/jm0306226.
13 Jackson PF, Slusher BS. Design of NAALADase inhibitors: a novel neuroprotective strategy. *Curr Med Chem*. 2001;8(8):949–957. https://doi.org/10.2174/0929867013372797.
14 Zhou J, Neale JH, Pomper MG, Kozikowski AP. NAAG peptidase inhibitors and their potential for diagnosis and therapy. *Nat Rev Drug Discov*. 2005;4(12):1015–1026. https://doi.org/10.1038/nrd1903.
15 Klusák V, Bařinka C, Plechanovová A, et al. Reaction mechanism of glutamate carboxypeptidase II revealed by mutagenesis, X-ray crystallography, and computational methods. *Biochemistry*. 2009;48(19):4126–4138. https://doi.org/10.1021/bi900220s.
16 J. Pavlicek J. Ptacek C. Barinka Glutamate carboxypeptidase II: an overview of structural studies and their importance for structure-based drug design and deciphering the reaction mechanism of the enzyme Curr Med Chem. 19 9 2012 1300 1309 CDT-EPUB-20120203-004 [pii].

17 Pomper MG, Musachio JL, Zhang J, et al. 11C-MCG: synthesis, uptake selectivity, and primate PET of a probe for glutamate carboxypeptidase II (NAALADase). *Mol Imaging*. 2002;1(2):96–101. https://doi.org/10.1162/153535002320162750.

18 Wüstemann T, Bauder-Wüst U, Schäfer M, et al. Design of internalizing PSMA-specific glu-ureido-based radiotherapeuticals. *Theranostics*. 2016;6(8):1085–1095. https://doi.org/10.7150/thno.13448.

19 Kozikowski AP, Nan F, Conti P, et al. Design of remarkably simple, yet potent urea-based inhibitors of glutamate carboxypeptidase II (NAALADase). *J Med Chem*. 2001; 44(3):298–301. https://doi.org/10.1021/jm000406m.

20 Chen Y, Pullambhatla M, Foss CA, et al. 2-(3-{1-carboxy-5-[(6-[18F]fluoro-pyridine-3-carbonyl)-amino]- pentyl}-ureido)-pentanedioic acid, [18F]DCFPyL, a PSMA-based PET imaging agent for prostate cancer. *Clin Cancer Res*. 2011;17(24): 7645–7653. https://doi.org/10.1158/1078-0432.CCR-11-1357.

21 Giesel FL, Hadaschik B, Cardinale J, et al. F-18 labelled PSMA-1007: biodistribution, radiation dosimetry and histopathological validation of tumor lesions in prostate cancer patients. *Eur J Nucl Med Mol Imaging*. 2017;44(4):678–688. https://doi.org/10.1007/s00259-016-3573-4.

22 Kularatne SA, Zhou Z, Yang J, Post CB, Low PS. Design, synthesis, and preclinical evaluation of prostate-specific membrane antigen targeted 99mTc-radioimaging agents. *Mol Pharm*. 2009;6(3):790–800. https://doi.org/10.1021/mp9000712.

23 Barinka C, Starkova J, Konvalinka J, Lubkowski J. A high-resolution structure of ligand-free human glutamate carboxypeptidase II. *Acta Crystallogr Sect F Struct Biol Cryst Commun*. 2007;63(3):150–153. https://doi.org/10.1107/S174430910700379X.

24 Hlouchova K, Barinka C, Konvalinka J, Lubkowski J. Structural insight into the evolutionary and pharmacologic homology of glutamate carboxypeptidases II and III. *FEBS J*. 2009;276(16):4448–4462. https://doi.org/10.1111/j.1742-4658.2009.07152.x.

25 Novakova Z, Cerny J, Choy CJ, et al. Design of composite inhibitors targeting glutamate carboxypeptidase II: the importance of effector functionalities. *FEBS J*. 2016;283(1):130–143. https://doi.org/10.1111/febs.13557.

26 Barinka C, Byun Y, Dusich CL, et al. Interactions between human glutamate carboxypeptidase II and urea-based inhibitors: structural characterization. *J Med Chem*. 2008;51(24):7737–7743. https://doi.org/10.1021/jm800765e.

27 Wu LY, Anderson MO, Toriyabe Y, et al. The molecular pruning of a phosphoramidate peptidomimetic inhibitor of prostate-specific membrane antigen. *Bioorganic Med Chem*. 2007;15(23):7434–7443. https://doi.org/10.1016/j.bmc.2007.07.028.

28 Majer P, Jackson PF, Delahanty G, et al. Synthesis and biological evaluation of thiol-based inhibitors of glutamate carboxypeptidase II : discovery of an orally active GCP II inhibitor. *J Med Chem*. 2003;46:1989–1996. https://doi.org/10.1021/jm020515w.

29 Krężel A, Maret W. The biological inorganic chemistry of zinc ions. *Arch Biochem Biophys*. 2016;611:3–19. https://doi.org/10.1016/j.abb.2016.04.010.

30 Dhumane NR, Hussaini SS, Dongre VG, Shirsat MD. Influence of glycine on the nonlinear optical (NLO) properties of zinc (tris) thiourea sulfate (ZTS) single crystal. *Opt Mater (Amst)*. 2008;31(2):328–332. https://doi.org/10.1016/j.optmat.2008.05.002.

31 Jakob U, Eser M, Bardwell JCA. Redox switch of Hsp33 has a novel zinc-binding motif. *J Biol Chem*. 2000;275(49):38302–38310. https://doi.org/10.1074/jbc.M005957200.

32 Klug A, Schwabe J. Zinc Fingers. *FASEB J*. 1995;9(8):597–604. https://doi.org/10.1096/fasebj.9.8.7768350.

33 Maddani MR, Prabhu KR. A concise synthesis of substituted thiourea derivatives in aqueous medium. *J Org Chem*. 2010;75(7):2327–2332. https://doi.org/10.1021/jo1001593.

34 Haitinger L. Vorläufige mittheilung über glutaminsäure und pyrrol. *Monatshefte für Chemie und verwandte Teile anderer Wissenschaften*. 1882;3:228–229.

35 Kumar A, Bachhawat AK. Pyroglutamic acid: throwing light on a lightly studied metabolite. *Curr Sci*. 2012;102(2):288–297. https://doi.org/10.2307/24083854.

36 Kampmeier F, Williams JD, Maher J, Mullen GE, Blower PJ. Design and preclinical evaluation of a 99mTc-labelled diabody of mAb J591 for SPECT imaging of prostate-specific membrane antigen (PSMA). *EJNMMI Res*. 2014;4(1):13. https://doi.org/10.1186/2191-219X-4-13.

37 Benešová M, Schäfer M, Bauder-Wüst U, et al. Preclinical evaluation of a tailor-made DOTA-conjugated PSMA inhibitor with optimized linker moiety for imaging and endoradiotherapy of prostate cancer. *J Nucl Med*. 2015;56:914–920. https://doi.org/10.2967/jnumed.114.147413.

38 Kalliokoski T, Kramer C, Vulpetti A, Gedeck P. Comparability of Mixed IC50 Data - A Statistical Analysis. *PLoS ONE*. 2013;8(4). https://doi.org/10.1371/journal.pone.0061007.

39 Kunchur NR, Truter MR. A detailed refinement of the crystal and molecular structure of thiourea. *J Chem Soc*. 1958:2551–2557. https://doi.org/10.1039/JR9580002551.

40 P. Vaughn J. Donahue The structure of urea. Interatomic distances and resonance in urea and related compounds Acta Crystallogr. 1952;(5):530–535. 10.1107/S0365110X52001477.

41 Tamames B, Sousa SF, Tamames J, Fernandes PA, Ramos MJ. Analysis of zinc-ligand bond lengths in metalloproteins: Trends and patterns. *Proteins*. 2007;69(3):466–475. https://doi.org/10.1002/prot.21536.