Structure, activity and metabolism of human glutamate carboxypeptidase II

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1 ABBREVIATIONS
ALS  amyotrophic lateral sclerosis
AMPA  alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
Beta-NAAG  N-acetyl-beta-aspartyl-glutamic acid
DM1  derivate of maytansine (from ethiopian plant Maytenus serrata),
tumor-activated immunotoxin
DNA  deoxyribonucleic acid
EGTA  ethylene glycol tetraacetic acid
FISH  Fluorescent in situ hybridization
FOLH1  folate hydrolase 1
GABA  \( \gamma \)-aminobutyric acid
GCPII  glutamate carboxypeptidase II
GCPIII  glutamate carboxypeptidase III
IC_{50}  median Inhibition Concentration
K_M  Michaelis-Menten constant
K_I  inhibition constant
LNCaP  lymph node carcinoma of the prostate
mAb  monoclonal antibody
mGluR  metabotropic glutamate receptors
mRNA  messenger ribonucleic acid
NAA  N-acetyl-aspartate
NAAG  N-acetyl-aspartyl-glutamate
NAALADase  N-acetylated-alpha-linked acidic dipeptidase
NAALADase L  N-acetylated-alpha-linked acidic dipeptidase-like protein
NMDA  N-methyl-D-aspartic acid
NMDAR  NMDA receptor
PAP  prostatic acid phosphatase
PCP  phenylcyclohexylpiperidine
PSA  prostate specific antigen
PSCA  prostate stem cell antigen
PSM'  truncated form of prostate specific membrane antigen at N-terminus
PSMA  prostate specific membrane antigen
PSM-C  alternatively spliced variant of PSMA
PSM-D  alternatively spliced variant of PSMA
PSM-E  alternatively spliced variant of PSMA
PSM-F  alternatively spliced variant of PSMA
2-PMPA  2-(phosphonomethyl)pentanedioic acid
QM/MM  quantum mechanics/molecular mechanics
rRNA  ribosomal RNA
RT-PCR  reverse transcriptase-polymerase chain reaction
TGF-\( \beta \)  transforming growth factor beta
2 PREFACE

---------------------------------------------------------------
Glutamate carboxypeptidase II (GCPII) is a type II transmembrane glycoprotein. Its expression was independently discovered in brain, prostate and also in small intestine. In the brain it is known as a N-acetylated-\(\alpha\)-linked acidic dipeptidase (NAALADase), in the prostate as a Prostate-Specific Membrane Antigen (PSMA), in the small intestine as a folate hydrolase (FOLH1). Although NAALADase, PSMA and FOLH1 were identified as a single protein at the end and the name of the protein was standardized to glutamate carboxypeptidase II (GCPII), the researchers from different disciplines use all four known names.

GCPII is a metallopeptidase from the M28 family. It is homologous to aminopeptidases (from *Aeromonas proteolytica* and *Streptomyces griseus*), which also belong to this family. The first model of domain representation was constructed based on this homology. The first X-ray structure was determined almost one decade later. The structure of extracellular part of GCPII reveals a symmetric homodimer with each monomer containing three domains (analogous to three domains of transferrin receptor): a protease domain, an apical domain, and a helical domain. Amino acids from all three domains are involved in substrate binding. Even though GCPII protein was also crystallized with inhibitors and product of hydrolysis (glutamate), the structure with a natural substrate has not yet been determined.

GCPII is expressed in high concentration in human brain. It hydrolyzes a substrate N-acetyl-aspartyl-glutamate (NAAG), which is cleaved into N-acetyl-aspartate and glutamate. NAAG is an abundant neurotransmitter in central nervous system and together with glutamate plays a role in excitotic neurotransmission. Inhibition of NAAG hydrolysis by specific and potent inhibitor 2-(phosphonomethyl)pentanedioic acid (2-PMPA) showed to be neuroprotective in animal models of stroke and neuropathic pain.

Interestingly enough, experiments with GCPII knock-out mice showed that GCPII is not the only protein with NAAG-hydrolyzing activity in the brain. It was shown that at least two homologous enzymes can be detected in mice: GCPII and GCPIII. They have similar pharmacological properties and affinity for NAAG.

GCPII is also overexpressed in prostate cancer and it is used as a new prostate cancer marker. Moreover, it is intensively studied as a possible therapeutic target because it is anchored in the membrane and it is expressed in all prostate cancers and also in neovasculature of several non-prostatic solid tumors. In the benign prostate, a truncated form (PSM’) of GCPII is prevalent. The ratio GCPII/PSM’ increases with the prostate cancer progression and can be used for prostate cancer prognosis (tumor index).
The study of GCPII is such a broad field that also this thesis is not focused on only one aspect of this interesting protein. Published papers in this thesis are reflection of this fact.

Beginning of this thesis deals with the structure of GCPII protein, to the architecture of its active site and protein folding. Moreover, it also addresses the structural features of several inhibitors of GCPII.

Second part deals with GCPII as a biological target. It is aimed at expression of GCPII in human brain and at the direct comparison between GCPII and its close homolog GCPIII. Moreover, it also concerns the study of GCPII and PSM' trafficking in cells and the origin of PSM'.

The thesis is written as an introduction and an overview of the published papers and manuscript, which are included in the thesis. The Methods and Material part is omitted because it is described in details in the enclosed papers. In the Results and Discussion background information and summary of individual papers are presented.
3 INTRODUCTION
3.1 GCPII AS AN INTERESTING MOLECULE (a general information)

3.1.1 GCPII: from DNA to protein

DNA

GCPII gene (FOLH1) spans 62,035 base pairs, consisting of 18 exons and 19 introns. All intron-exon boundaries conform to the GT-AG rule [1], thus there is space for rich alternative splicing. Using FISH\(^1\) analysis, the location of GCPII was found in chromosome 11, at p11.2 arm [2, 3].

GCPII promoter lies upstream of GCPII encoding gene FOLH1. It contains number of potential sites for transcription factor binding, none of which by itself is responsible for such a high expression of GCPII in prostate [1, 4].

GCPII enhancer is in the third intron. It contains 72 bp direct repeat within a 331 bp core region and activates transcription from its own and heterologous promoters in prostate cell lines. The enhancer is repressed in the presence of androgen [5, 6].

mRNA

GCPII mRNA offers various sites for alternative splicing. Until recently, three alternatively spliced forms were discovered and designated as PSM' [7, 8], PSM-C and PSM-D (Fig. 1) [7] and also variants PSMA\(_6\) [9] and PSMA\(_{18}\), in which complete exon 6 or 18 are excluded, respectively [10]. Recently, another two new forms of alternative splicing were found: PSM-E [11] and PSM-F in the database (on the web sites of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) in Protein: sequence database).

\(^{1}\) Glossary item
Fig. 1: cDNA sequences of GCPII and three alternatively spliced variants (PSM’, PSM-C and PSM-D). **Panel A**: In PSM’ bases 114-380 (pale blue box in GCPII) of genomic GCPII sequence are removed by alternative splicing. PSM-C has the same splice donor site as PSM’ (nucleotide 114) but alternative acceptor site is located in intron one of GCPII; mRNA contains additional 133 nucleotides (green box). PSM-D has the same splice donor site as PSM’ and an unique acceptor site within intron one. It includes a novel exon (orange box) [7]. **Panel B**: Translation of GCPII mRNA yeilds a 750 amino acids long protein consisting of intracellular domain (IN), transmembrane domain (TM) and large extracellular domain (EXT). PSM’ protein lacks 59 amino acids from N-terminus compared to GCPII (full length form). PSM-C protein is predicted to be identical to PSM’. PSM-D protein is predicted to be a protein with novel 21 amino acids on the N-terminus (red box).

PSM’ is the most often described alternatively spliced variant. It lacks 266 nucleotides near 5’ end and the translated protein has no intracellular and transmembrane domain. It was detected only in the prostate and in no other tissue [8]. Its importance in normal prostate and prostate cancer will be discussed elsewhere (see chapter: 3.3.1.2.3.4.2).

Translation of PSM-C mRNA variant would yeild protein identical to PSM’. Translation of PSM-D mRNA would yield a protein of 97kDa, without transmembrane domain and with different amino acids at N-terminus of the protein compared to GCPII [7].

**PROTEIN**

Glutamate carboxypeptidase II (EC 3.4.17.21) belongs to the M28 family of metallopeptidases on the basis of sequence homology to the aminopeptidases of this family.

GCPII is a membrane type II glycoprotein with short intracellular N-terminus and large extracellular domain containing active site and possessing hydrolytic activity [12]. It is a 750 amino acids long protein with molecular weight of 100kDa. The structure of GCPII will be discussed later (see chapter 3.1.5).
3.1.2 GCPII hydrolytic activities

3.1.2.1 N-acetylated-alpha-linked acidic dipeptidase activity

GCPII is able to cleave N-acetyl-aspartyl-glutamate (NAAG), a neurotransmitter (see chapter 3.2.1.2) (Fig. 2) [13]. It cleaves off the C-terminal glutamate and thus releases it into the synapse. Biological significance of this cleavage is discussed in detail elsewhere (see chapter 3.2 GCPII as a neuropeptidase).

![Fig. 2: Hydrolysis of the substrate N-acetyl-aspartyl-glutamate (NAAG) by GCPII to yield N-acetyl-aspartyl (NAA) and neurotransmitter glutamate (Glu).](image)

3.1.2.2 Folylpoly-gamma-glutamate carboxypeptidase activity

This activity was characterized in the brush border of intestinal mucosa and converts folylpoly-gamma-glutamate into the folate (Fig. 3) that is ready for intestinal uptake [14]. This activity is responsible for cleavage of methotrexatepolyglutamate and thus it is important for its uptake and metabolism [15]. Due to this activity, GCPII can also be a target for activation of peptide prodrug in prostate cancer. Methotrexate-based peptide analogs were analysed to identify new GCPII-specific substrate. However, the longer γ-linked analogs of methotrexate were not significantly hydrolysed into methotrexate. Moreover, these analogs were transported into cell via folate transport mechanism independent of GCPII hydrolysis and thus might not be preferred agents for targeting GCPII activity [16].
3.1.3 GCPII substrate specificity

Generally, dipeptides are much better substrates than those with longer chain, alpha-linked peptides are better than gamma-linked and peptides containing C-terminal glutamate are the best substrates of all [13]. Using libraries of all possible dipeptides, new substrates for GCPII were discovered [17]. They were identified as: Ac-Glu-Met, Ac-Asp-Met, Ac-Ala-Glu, and Ac-Ala-Met, yet dipeptides containing Met as a C-terminal amino acid showed two order of magnitude loss in binding to GCPII (Table 1) [17].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_M$ [µM]</th>
<th>$k_{cat}$ [s$^{-1}$]</th>
<th>$k_{cat}/K_M$ [s$^{-1}$/mmol$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-Asp-Glu</td>
<td>0.43 ± 0.1</td>
<td>0.59 ± 0.16</td>
<td>1372</td>
</tr>
<tr>
<td>Ac-Glu-Glu</td>
<td>&lt;5.0</td>
<td>0.78 ± 0.08</td>
<td>ND</td>
</tr>
<tr>
<td>Ac-Ala-Glu</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ac-Glu-Met</td>
<td>53.0 ± 5.9</td>
<td>0.29 ± 0.01</td>
<td>5.5</td>
</tr>
<tr>
<td>Ac-Asp-Met</td>
<td>24.8 ± 3.9</td>
<td>0.07 ± 0.002</td>
<td>2.8</td>
</tr>
<tr>
<td>Ac-Ala-Met</td>
<td>303 ± 41</td>
<td>0.01 ± 0.001</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Table 1: Example of kinetic parameters of GCPII for different dipeptides from peptide libraries. ND, not determined. Modified from [17].

3.1.4 GCPII inhibitors

GCPII is inhibited by polyvalent anions such as phosphate, sulphate [13], and by compounds chelating divalent cations (like EGTA), which is a common feature of metallopeptidases. Interestingly enough, GCPII activity was also inhibited by bestatin and puromycin (aminopeptidase inhibitors) [13].

Studying derivates of NAAG and glutamate and their inhibition efficiency on GCPII revealed effective inhibitors N-succinyl-Glu-OH, N-fumaryl-Glu-OH ($K_I$ 0.9 and 0.4 µM, respectively) (Fig. 4) [18], noncompetetive inhibitor quisqualic acid and competitive inhibitor beta-NAAG (N-acetyl-aspartyl-beta-linked-glutamate) (Fig. 4) [19].
In the panel of hydroxyphosphinyl derivates [20] and urea-based inhibitors of GCPII [21], the most potent inhibitor of GCPII 2-(phosphonomethyl)pentanedioic acid (2-PMPA) (K_i of 300pM) (Fig. 4) was discovered [20]. Further analysis showed that for a good inhibition potency the inhibitor should contain acidic moiety in propionic acid portion. None of the new designed inhibitors was so efficient as 2-PMPA [22].

Practical application of GCPII inhibition as a therapeutical tool (see chapter 3.2 GCPII as a neuropeptidase), requires efficient orally bioavailable inhibitor and because 2-PMPA meets only one of these criteria (efficiency), 2-(thioalkyl)pentanedioic acids were synthesised. The inhibition by these compounds was dependent on the number of methylene units between the thiol group and pentanedioic acid. Although none of these compounds are as efficient as 2-PMPA, very potent inhibitor 2-(3-mercaptopropyl)pentanedioic acid (2-MPPA, IC_50 90nM) (Fig. 4) was found to be orally bioavailable in rats and showed efficacy in an animal model of neuropathic pain following oral administration [23]. These thiol based inhibitors were further modified to contain at the P1’ position benzyl moiety. 3-(2-carboxy-5-mercaptopentyl)-benzoic acid (IC_50 15nM) (Fig. 4) was more potent than 2-MPPA. However, the replacement of 2-carboxyethyl group with 3-carboxybenzyl group in 2-PMPA resulted in significant loss of inhibitor potency [24].
3.1.5 GCPII structure

3.1.5.1 Overall structure of GCPII

The structure of extracellular part of GCPII reveals a symmetric homodimer with each monomer containing three domains (analogous to three domains of transferrin receptor): a protease domain (residues 57-116 and 352-590), an apical domain (residues 117-351), and a helical domain (residues 591-750) [25, 26]. Amino acids from all three domains are involved in substrate binding. The dimer interface of GCPII is large (total of 2457 Å²) [26], suggesting
thus that monomer would dissociate from GCPII only upon partial unfolding and perhaps that is why the only active species of GCPII in vitro seems to be a dimer [27] (Fig. 5).

The protease domain is most closely related to the domain of aminopeptidase from *Aeromonas proteolytica* [25, 26]. The domain contains a central seven-stranded mixed β-sheet surrounded by ten α-helices [26].

![Three-dimensional representation of GCPII dimer](image)

**Fig. 5: Three-dimensional representation of GCPII dimer.** One monomer is shown in wheat colour, the other is coloured according to domain organisation. In the active site there are zinc ions (blue spheres), calcium ions (yellow spheres), and also chloride ions (orange spheres). Seven carbohydrate side chains located in the electron density map are shown in line representation. N- and C-terminus of this extracellular domain is labeled as N and C, respectively.

Inserted between first and second strand of the central β-sheet of the protease domain is the apical domain. It covers the active site and creates a substrate binding funnel between the domains [26] (Fig. 5).

The helical (or C-terminal) domain has a main feature consisting of up-down-up-down four-helices bundle. There are also two loops; first one (residues 676-690) creates contact with protease domain through hydrogen bonds. The second loop (residues 692-704), which also makes a several hydrogen bonds to protease domain, is a part of the glutarate
sensor“. „Glutarate sensor“ is a hairpin containing residues Lys699 and Tyr700, which are directly involved in the specific substrate binding. This second loop is flexible and its conformation changes in the crystal structures depending on ligand bound in the S1’ position of the active site [26, 28] (Fig. 6, panel A, B). Helical domain also forms a dimer interface, which comprise mostly of helical domain of one monomer and protease and apical domains of the other. There are also two intermolecular salt-bridges formed between Arg662 of one monomer in helical domain and Asp666 of helical domain of other monomer [26].

**Fig. 6:** „Glutarate sensor“ in the GCPII structure. Amino acid residues 692-704 create a flexible loop that is involved in substrate binding. **Panel A:** The GCPII structures available from the RSCB PDB were superimposed. The Cα-traces are shown in ribbon representation. Colour-coding used for „glutarate sensor“ is depicted in cyan for GCPII/GPI-18431 (GCPII inhibitor) structure, in yellow for GCPII/glutamate structure and in magenta for...
GCPII/phosphate structure. Residues Lys699 and Tyr700 create a part of this loop. They directly bind ligands in S1’ site. If the S1’ site is not occupied by a ligand (structure GCPII/phosphate; magenta), the loop change its position in the active site. **Panel B**: Detailed picture of “glutarate sensor”. Different position of the loop highlights the movement of Lys699 and Tyr700 (in line representation) in the structure of GCPII/phosphate. Colour-coding is the same as in panel A.

In the GCPII structure a calcium ion is also located. Two domains (protease and apical) co-operate in its binding. The Ca$^{2+}$ is too distant from active site to be involved in enzyme catalysis. Its role is more likely to hold protease and apical domain together through coordinative interaction. Possibly, it is involved in dimerization by stabilizing the loop 272-279 which has three tyrosins (272, 277, 279) (Fig. 7). These tyrosins form hydrophobic pocket, which is entered by side chain of Tyr733 of the other monomer in the dimer (Fig. 7). Moreover, Tyr277 forms an intermolecular hydrophobic interaction with N-acetyl group of N-acetyl-glucosamine of the sugar chain attached to Asn638. This contact of sugar chain and protease domain is one of the few structurally well-defined cases of a protein–carbohydrate contact involved in homodimerization of a protein (Fig. 5) [26].

**Fig. 7: Function of Ca$^{2+}$ ion is still not understood.** Ion Ca$^{2+}$ is coordinated by two glutamates and threonine. Three tyrosins (in gray colour) in one monomer form a hydrophobic pocket, which is entered by side chain of Tyr733 of the other monomer in the dimer (depicted in orange). This interaction can play a role in dimerization. Monomers of GCPII dimer are coloured in green and wheat.

Another ion in the GCPII structure is Cl$^{-}$ ion. It seems that it has stabilizing role and holds Arg534 in favorable conformation for substrate binding [26].
3.1.5.2 Active site of GCPII

Approximately 20Å long funnel leads from the GCPII surface to the active site that contains two zinc ions. One zinc ion (Zn1) is tetrahedrally coordinated by Glu425, His553 and by a bridging ligand Asp387. This aspartate also coordinates other zinc ion (Zn2) together with Asp453 and His377. The catalytic zinc center in free state has a single water molecule bridging two zinc ions (Fig. 8). Interestingly, the distance between zinc ions is changing from 3.30Å, in the free state, to 3.65 and 3.78Å, respectively, in the complexes with phosphate and GPI-18431 [26].

Asp387 in the active site forms a peptide bond in cis conformation with its neighbouring Pro388, which is common in binuclear zinc peptidases [26].

Fig. 8: Active site of GCPII. Zinc ions are depicted in blue. Each metal ion is tetrahedrally coordinated by histidine, acidic amino acid and by a bridging ligand Asp387. Glu424, a possible catalytic acid/base of GCPII, is also depicted.

In the catalytic mechanism Glu424 plays a prominent role. It is a probable catalytic acid/base of GCPII. As a proton shuttle it abstracts a proton from the water between zinc ions and transfers the proton to the amino group of glutamate, possibly during substrate cleavage. The activated water molecule then attacks carbonyl group of aspartate in the substrate (Fig. 9) [26]. This catalytic mechanism is similar to those discovered in other carboxypeptidases [29].
Fig. 9: The catalytic mechanism of GCPII. Catalytic pathway was suggested on the basis of proposed catalytic mechanism of aminopeptidases from Aeromonas proteolytica [29] and Streptomyces griseus [30]. Tyr552 can play an important role in stabilizing the transition state during catalysis (Tyr246 plays similar role in aminopeptidase from Streptomyces griseus [30]). Dashed lines indicate stabilizing interaction and/or hydrogen bond in the catalysis. Inserted panel A: a product of hydrolysis L-glutamate (green) is located in the active site together with Glu424 (green), Tyr552 (orange), and water molecule between two zinc ions (blue spheres). Inserted panel B: a catalytic acid/base Glu424 (in green) of GCPII is shown together with activated water molecule between two zinc ions (blue spheres).
3.2 GCPII AS A NEUROPEPTIDASE

The expression of GCPII is not as tissue-restricted as was expected a few years ago [31-37]. Yet there are still several tissues with higher GCPII expression. One of these tissues is the brain. The function of this enzyme is known there, contrary to other tissues. Moreover, GCPII seems to play a role in neuropathologies where excessive amount of glutamate was detected.

3.2.1 Glutamate, NAAG, GCPII and nervous system

3.2.1.1 Glutamate-excitatory neurotransmitter

Glutamate is primary excitatory neurotransmitter in the human nervous system and it plays role in neurodevelopment and, unfortunately, also in neurodegeneration. It acts postsynaptically on three ionotropic receptors (Table 2), named after their preferentially agonists\(^2\) [38]. These receptors have incorporated ion channels permeable for cations.

Glutamate also binds to metabotropic receptors (Table 2), which are linked to G-proteins\(^3\) and operate by releasing second messenger into the cytoplasm or influence ion channels through releasing G-protein subunits within the membrane [39].

There are also other ligands, except glutamate, which bind to the glutamate receptors [40-42]. Among them an endogenous dipeptide N-acetyl-aspartyl-glutamate (NAAG) can be found.

\(^{2,3}\) Glossary item
<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Group</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA</td>
<td>ionotropic</td>
<td></td>
<td>calcium influx into post-synaptic cell, a signal crucial for induction of LTP and LTD (long-term potentiation and long-term depression)</td>
</tr>
<tr>
<td>APMA</td>
<td>ionotropic</td>
<td></td>
<td>mediate fast synaptic transmission in the CNS</td>
</tr>
<tr>
<td>kainate</td>
<td>ionotropic</td>
<td></td>
<td>synaptically</td>
</tr>
<tr>
<td>mGlu1</td>
<td>metabotropic</td>
<td>I</td>
<td>activates adenylate cyclase; increases activity of phospholipase C; inhibits K⁺ channels</td>
</tr>
<tr>
<td>mGlu5</td>
<td>metabotropic</td>
<td>I</td>
<td>inhibits K⁺ channels</td>
</tr>
<tr>
<td>mGlu2</td>
<td>metabotropic</td>
<td>II</td>
<td>inhibits adenylate cyclase; inhibits voltage-gated Ca²⁺ channels; activates K⁺ channels</td>
</tr>
<tr>
<td>mGlu3</td>
<td>metabotropic</td>
<td>II</td>
<td>inhibits adenylate cyclase; inhibits voltage-gated Ca²⁺ channels; activates K⁺ channels</td>
</tr>
<tr>
<td>mGlu4</td>
<td>metabotropic</td>
<td>III</td>
<td>inhibits adenylate cyclase; inhibits voltage-gated Ca²⁺ channels</td>
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<td>III</td>
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</tr>
<tr>
<td>mGlu8</td>
<td>metabotropic</td>
<td>III</td>
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</table>

Table 2: Glutamate receptors. The classification of mGlu receptors is determined by their similarities in coupling mechanism, molecular structure, sequence homology and the pharmacology of the receptors.

### 3.2.1.2 N-acetyl-aspartyl-glutamate (NAAG)

NAAG is the most prevalent and widely distributed peptide neurotransmitter in the mammalian nervous system [43-45].

Specific antibodies against NAAG showed wide distribution of the peptide through the mammalian brain, spinal cord, sensory neurons [46-49], and mammalian retina [50-53]. NAAG seems to be strictly located in neurons containing a variety of amine neurotransmitters including glutamate, GABA [44], although, in glial cells cultures moderate micromolar concentrations of NAAG were also found [54]. It is concentrated into synaptic vesicles [55, 56] and released by depolarization-induced, calcium-dependent manner [57, 58]. NAAG is hydrolyzed (enzymatically inactivated) by glutamate carboxypeptidase II in the synaptic cleft [59]. However, it is also directly taken back to neurons [60].

NAAG novel receptor was identified by receptor binding studies, which showed binding of NAAG at NMDA receptor binding sites (IC₅₀ values: glutamate, 0.4 mM; NAAG, 8.8 mM) in rat forebrain membranes [61]. NAAG is a low-potency agonist² of NMDA receptors, but not kainate or AMPA receptors [62-64]. Interestingly enough, NAAG also acts as an antagonist⁴ of NMDA receptors at low concentration (below 20µM) [65-67].

NAAG was also detected as a selective agonist of mGluR3 receptors with a potency that rivaled glutamate [68, 69]. One role of NAAG in the nervous system is to activate

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²,⁴ Glossary item
presynaptic mGluR3 receptors and thus suppress synaptic release of itself and mainly glutamate [70]. It also activates postsynaptic mGluR3 and decreases the cAMP levels via inhibitory G protein in neuron and astrocytes (Table 2). The downstream consequences of this process are not well established [71, 72].

The NAAG neurotransmission action is summarized in Fig. 10.

![Illustration of the NAAG neurotransmission](image)

**Fig. 10: Illustration of the NAAG neurotransmission.** After release, at the post-synaptic membrane, NAAG attenuates excitability by competing with glutamate for binding at NMDA receptors or by the activation of postsynaptic mGluR3. At the pre-synaptic membrane, it activates inhibitory mGluR3 to attenuate further release of neurotransmitter, including glutamate and NAAG. NAAG is removed from the synaptic cleft by hydrolysis into glutamate and NAA catalyzed by GCPII (at astrocyte membrane). The reaction products are taken up by astrocytes. Glutamate and NAAG can be also taken back to pre-synaptic neuron.

### 3.2.1.3 NAAG peptidase (= glutamate carboxypeptidase II = GCPII)

GCPII cleaves N-acetyl-aspartyl-glutamate in the synaptic cleft into glutamate and N-acetyl-aspartate (NAA) (Fig. 10) and the reaction products can act in neurotransmission or can be taken back to pre-synaptic neurons or astrocytes.
The peptidase activity of GCPII is widely distributed throughout the nervous system, consistent with the distribution of NAAG [66, 73, 74]. NAAG peptidase activity has been located to extracellular space of plasma membrane of isolated retinal cells, cultures containing mouse brain neurons and glia 5, mouse brain glia cultured alone, and nonmyelinating Schwann cells 5 [54, 75-77]. GCPII is concentrated in glia, namely astrocytes 5 [78].

If NAAG is cleaved, glutamate is released and thus concentration of excitotoxic neurotransmitter is increasing. On the other hand, if GCPII is inhibited and NAAG is not cleaved, it binds to NMDA receptors [62, 64] and also to pre-synaptic metabotropic glutamate receptors, where it negatively influences releasing of glutamate into synapse [70].

Inhibitors of GCPII can be valuable therapeutic tool in neurological diseases, because they indirectly decrease excitotoxic glutamate. Moreover, they influence and enhance a natural regulatory process (decreasing glutamate) in contrast to other strategies, which chronically activate or inhibit receptors in a manner which is unrelated to ongoing neurotransmissions and which is almost always accompanied by side effects.

3.2.2 Acute neurological disorders and GCPII

3.2.2.1 Stroke

Ischemic injury is the cause of excitotoxic nerve-cell death which occurs in the area of insult and also in nervous tissues in surroundings. Excessive levels of glutamate, overstimulation of NMDA receptors and influx of Ca\(^{2+}\) into the cell are the major factor of nerve-cell death [79].

In 1999 Slusher et al. proposed a new strategy for the treatment of stroke by inhibition of GCPII. Use of selective inhibitor 2-(phosphonomethyl)pentanedioic acid (2-PMPA) (Fig. 4) showed to be neuroprotective in neuronal culture model of stroke and in rats after transient middle cerebral artery occlusion 6 [80].

Neuroprotection during ischemia is caused by inhibition of GCPII hydrolyzing activity (2-PMPA), increase of NAAG and decrease of glutamate, through the action of NAAG on the metabotropic glutamate receptors [80-82].

Moreover 2-PMPA does not induce the learning and memory deficit and neurotoxic properties of NMDA receptor antagonists and has no effect on glutamate levels in normal

\(^{5,6}\) Glossary item
non-ischemic rats [80]. The neuroprotective effects of 2-PMPA were also confirmed in peripheral neuropathies [83], retinal ganglion cell death caused by excessive glutamate receptors activation [84] and in spinal cord injury [85].

Another interesting discovery was that the neuroprotection mediated through GCPII inhibition appears to be dependent on the presence of glial cells in the neuronal/glial culture [86, 87]. Interestingly, increasing concentration of NAAG (caused by GCPII inhibition) can activate mGluR3 receptors, expressed by astrocytes, which control release of transforming growth factor beta (TGF-β) that have a neuroprotective effect in neurodegenerative disorders (Fig. 11) [88]. Neuroprotection was reversed by antagonist of mGluR3 receptor and also by use of neutralizing antibodies against TGF-β. Taken together, these results suggest involvement of glial mGlu receptors in NAAG and 2-PMPA mediated neuroprotection [86, 87].

**Fig. 11: The neuroprotective effect of transforming growth factor-β (TGF-β).** Activation of glial mGlu3 receptors causes the release of TGF-β, through the activation of mitogen-activated protein kinase (MAPK) and PI-3-K pathways. TGF-β released from astrocytes may exert its neuroprotective effect in glial cells by inducing an expression of the serpin PAI-1 (plasminogen activator inhibitor type 1), which is then released from the astrocyte (left panel), and by acting in neurons through a set of high affinity serine-threonine kinases (TGF-βRI and TGF-βRII), which signal to the nucleus through the activation of transcriptional factors (SMAD2/3, SMAD4) or through the activation of transforming growth factor kinase 1 (TAK1). Downstream events include the induction of cell cycle proteins (p15, p21, and p27) or the inhibition of cyclooxygenase-2 (COX-2) expression. Other factors, still unknown, might contribute to TGF-β–induced neuroprotection (right panel). Modified from [39].

7 Glossary item
3.2.2.2 Head injury

Traumatic brain injury\(^8\) is followed by secondary injury associated with excessive elevation of extracellular glutamate and following cell death occurs [89]. Neurons and glial cells in the hippocampus, a brain area associated with learning and memory, are exceptionally sensitive to trauma-induced excitotoxic cell death [90].

In an animal model of fluid percussion\(^9\), the inhibition of GCPII reduced glutamate concentration and the loss of degenerating neurons and astrocytes. Use of metabotropic glutamate receptor antagonists LY-341495 completely abolished the neuroprotecting activity of GCPII inhibition and probably effect of NAAG on mGlu3 receptors [91, 92]. The data support the hypothesis that GCPII inhibition might decrease magnitude and duration of excitotoxic events associated with brain injury and represent thus new therapeutic target in brain injury.

3.2.2.3 Neuropathic and inflammatory pain

Increased glutamate concentration in the spinal cord and primary afferent nerves\(^10\) plays an important role in acute and chronic pain. Ectopic discharges\(^11\) from afferent nerves in the site of nerve injury are responsible for the development of hypersensitivity, hyperalgesia\(^12\) and allodynia\(^13\) (abnormal pain sensation) in patients with painful neuropathies [93].

Mechanical allodynia induced in inflammatory model (formalin test or carrageenan injection\(^14\)) is mediated by spinal NMDA receptors-dependent mechanism [94]. Using low levels of selective GCPII inhibitor 2-PMPA (1-100μg) caused accumulation of small amount of NAAG, which acts as an antagonist on NMDA receptors and has an anti-allodynic effect (attenuating the level of mechanical allodynia). At higher doses (300μg) this effect disappears because of accumulation of larger amount of NAAG, which acts now as an agonist on NMDA receptors [95, 96, 97].

Even though there are two controversial studies about analgetic effect of GCPII inhibition through the NAAG effect on pre-synaptic mGlu receptors [98, 99], orally bioavailable GCPII inhibitor (2-(3-mercaptopropyl)pentanedioic acid (IC50 90nM); see Fig. 4) exhibited efficiency in an animal model of neuropathic pain [23].

\(^8\)-\(^{14}\) Glossary item
3.2.3 Chronic neurological disorders and GCPII

3.2.3.1 Schizophrenia

Schizophrenia is a chronic brain disorder where alternations in dopaminergic and glutamatergic transmission seem to play an important role [100-102]. One theory of schizophrenia is the glutamate theory, which involves hypofunction of the glutamate neurotransmitter system where also NMDA and mGluR II receptors belong. [103-105]. Even though NAAG acts on both these receptors and GCPII inhibition seems to be interesting therapeutical approach, all studies published to date are inconclusive [102, 106, 107].

3.2.3.2 Diabetic neuropathy

Diabetic neuropathy\(^\text{15}\) is a common complication of diabetes. It damages the nerves that allow to feel sensations such as pain [108, 109]. Glutamate excitotoxicity is believed to be a prominent mechanism involved in diabetic neuropathy [110].

GCPII inhibitor (2-PMPA) in model of diabetic neuropathy showed that the prevention of glucose-induced programmed cell death and positive effect on neuropathic hyperalgesia, nerve dysfunction of both myelinated and unmyelinated fibers, was specific and was mediated by NAAG agonist activity on mGluR3 receptor [111, 112].

3.2.3.3 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal degeneration of motor neurons in the spinal cord and cerebral cortex [113]. Glutamate excitotoxicity has been implicated as a mechanism of motor neuron death in this disease [114].

In ALS patient, NAAG levels are elevated [114] and in addition GCPII activity is upregulated [115]. The inhibition of GCPII by 2-PMPA and 2-MPPA decreased death of motor neurons and glia in the animal model of ALS. This neuroprotective effect is mediated through the limiting glutamate levels (originating from NAAG) when glutamate uptake is abnormal [116].

\(^\text{15}\) Glossary item
3.3 GCPII AS A DIAGNOSTIC MARKER AND THERAPEUTIC TARGET IN THE PROSTATE CANCER

GCPII is overexpressed in prostate cancer and it is used as a new prostate cancer marker with its pros and cons. Moreover, it is studied as a possible therapeutic marker of the prostate because it is anchored in the membrane and it is expressed in all prostate cancers and also in neovasculature of several non-prostatic solid tumors.

3.3.1 GCPII as a marker of prostate cancer

3.3.1.1 Prostate cancer in numbers

Prostate cancer is the second most prevalent cancer in the Czech Republic. At least in 15% of men over age 50 oncogenic changes in prostate tissue were detected. Worldwide occurrence of this cancer is increasing by about 3% every year.

Czech Republic takes 40th place in the incidence of prostate cancer (source from Epidemiology of malignant tumors in the Czech Republic; www.svod.cz [117]).

Graph 1: Time trend of crude incidence (number of new cases per 100 000 persons) and crude mortality (number of deaths on a diagnosis per 100 000 persons); (source from http://www.svod.cz).

It can be seen that the incidence and mortality is still increasing (Graph 1). Individuals with prostate cancer lose 8-9 years of their life span and although this type of cancer is sometimes called an indolent and incidental cancer, only 70% of prostate cancer patients have 10-year survival compared to general population [118].
With the aging population the incidence of the prostate cancer increases. However, the increased incidence can be partially attributed to novel methods in earlier detections.

The tumors of epithelial cells belong to the most common type of prostate cancer. The leading two tumors are: carcinoma\textsuperscript{16} of the prostate and benign hyperplasia\textsuperscript{17} (malignant tumor of epithelial cells and benign adenoma, respectively).

The majority of prostate carcinoma represents adenocarcinomas\textsuperscript{18} from prostatic epithelial cells (over 95%) \cite{119}.

### 3.3.1.2 Markers of the prostate cancer

Some tumor markers have diagnostic values and we call them diagnostic markers. They distinguish cancerous disease from other abnormalities in the prostate. Some markers have prognostic values: they are called prognostic markers; they should independently predict the biological behavior and outcome of prostate cancer \cite{118}.

#### 3.3.1.2.1 Prostatic Acid Phosphatase (PAP)

It is a non-specific phosphomonoesterase secreted into seminal plasma under androgen control \cite{120}. PAP represents only a small portion of the total acid phosphatases in the serum of normal men, thus cross reactivity during screening is observed with serum acid phosphatases. It is also not a prostate specific marker; it is synthesized by granulocytes, spleen and pancreas \cite{121}. Limited ability to detect early prostate cancer, high false negative results and elevation of PAP in already advanced disease, lead to current replacement of PAP by prostate specific antigen.

#### 3.3.1.2.2 Prostate specific antigen (PSA)

It is a serine protease secreted by prostate epithelial cells and it is a normal component of the ejaculate \cite{122-124}. PSA was detected in serum of men with benign and malignant prostatic diseases and it is widely used as a tumor marker. Even though it was believed to be prostate specific, it was also detected in other body tissues \cite{125-129}, cancers of non-prostatic origin \cite{130, 131} and also in serum of normal women \cite{130-132}.

\textsuperscript{16-18} Glossary item
PSA testing is used normally in hospitals as a standard test [133] for prostate cancer patients and detects pathologically organ-confined prostate cancer in large number of cases. Nevertheless, it is not disease-specific, elevated levels can be find in a small portion of normal males and in 25-85% of patients with benign prostatic hyperplasia (BPH) and prostatitis [134]. Moreover, PSA serum levels are downregulated by the absence of testosterone (stimulates cancer cell growth) during androgen-deprivation therapy, which is normally used for reducing the male hormones [135]. Thus it makes the diagnosis more difficult.

3.3.1.2.3 GCPII disguised as Prostate specific membrane antigen

An ideal diagnostic tumor marker SHOULD [118]:

- Meet general tumor marker criteria but also be an organ-specific and cancer-specific
- Have high sensitivity
- Have high specificity and reproducibility
- Be practical; simple, cost-efficient test
- Predict the prognosis

AN IDEAL PROSTATE CANCER MARKER HAS NOT YET BEEN DISCOVERED.

3.3.1.2.3.1 GCPII organ-specificity

Although GCPII is mostly expressed in brain, prostate, kidney and small intestine [31, 33, 35, 136, 137], it was also detected in other organs [32, 36]. It is very disturbing that different studies very often show different results of tissue localization of the enzyme (Table 3). In many examples 7E11 antibody, which recognizes cytosolic N-terminus of GCPII, was used [31-33, 35-37]. Only in half of these studies new antibodies against extracellular part of GCPII were applied [33, 34, 37, 138, 139]. Also the methods of protein detection are different and could have an influence on final results (Table 3).

GCPII does not seem to be located strictly into the prostate tissue, it can be found in many other normal tissues through the human body. But there are only three tissues (kidney proximal tubules, prostate and duodenum) in which all studies showed consistent results (Table 3). It is not surprising since these tissues express high concentration of GCPII and thus
even antibodies with low sensitivity can detect the protein. Interestingly, three studies did not observe expression in human brain [35, 36, 138], although there are several other studies, which agree with each other in positive expression of GCPII protein in human brain [33, 137, 139, 140]. Moreover, the brain localization was also confirmed by activity measurement [141].

Even though the results of tissue localization are not consistent (because of different antibodies and detection methods used), it can be concluded that GCPII is not strictly prostate specific (Table 3).

Fig. 12: GCPII staining in different human tissues. A. Benign prostate tissue, weak to moderate GCPII staining, apical to cytoplasmic pattern. B. Testis; cytoplasmic staining. C. Urothelium of the urinary bladder; cytoplasmic staining. D. Brain tissue; cytoplasmic staining. Adopted from [139].
<table>
<thead>
<tr>
<th>tissue</th>
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<tbody>
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<td>bone marrow</td>
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<td>brain</td>
<td>Chang et al. [138] (^2)</td>
</tr>
<tr>
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<td>Dumas et al. [31] (^3)</td>
</tr>
<tr>
<td>capillaries</td>
<td>Gala et al. [32] (^2)</td>
</tr>
<tr>
<td>cardiac muscle</td>
<td>Sokoloff et al. [37] (^3)</td>
</tr>
<tr>
<td>cerebellum</td>
<td>Kinoshita et al. [33] (^1)</td>
</tr>
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<td>Mhawech-Fauceglia et al. [139] (^2,4)</td>
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Table 3: GCPII expression in different human tissues. Y, protein expression detected. 0, no expression detected. 1, western blot; 2, immunohistochemistry; 3, dual-monoclonal sandwich assay; 4, tissue microarray. Inserted pictures: immunohistochemistry of A, prostatic tissue, adopted from [139] and B, urothelium, adopted from [32]. Different studies used different antibodies. 7E11-C5.3 (widely used as 7E11) applied: [31-33, 35-37, 137, 138]. J591: [34, 138]. J415: [138]. PEQ226.5: [37, 138]. PM2J004.5: [138]. 24.4E6: [33]. Y-PSMA-1: [139].
3.3.1.2.3.2 GCPII cancer-specificity

Results from GCPII expression in malignant tissues are summarized in Table 4.

New study concerning detection in normal (Table 3) and malignant (Table 4) tissues was published recently [139]. This study analysed 3161 benign and malignant tumors for GCPII presence. GCPII was expressed in 154 (5.4%) from 2174 malignant tumors. The only brain tumor positive tissue for GCPII was glioblastoma multiforme in 3/148 of brain cases overall. And finally all 846 benign tumors were negative for GCPII [139].

Fig. 13: GCPII expression in prostate adenocarcinoma. A. Apical pattern. B. Apical/cytoplasmic. C. Cytoplasmic with membranous accentuation. D. Cytoplasmic only. Adopted from [139].
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<td>10</td>
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Table 4: Expression of GCPII in different malignant tissue. Y, protein expression detected. 0, no expression detected. Methods used for GCPII determination are the same as in protein detection from normal tissues. 1, Chang et al. [142] used immunohistochemistry, and antibodies 7E11, J591, J415. 2, Chang et al. [138] used immunohistochemistry, and antibodies 7E11, PM2J004.5, PEQ226.5, J591, J415.
Moreover, the neovasculature of several solid tumors was also GCPII positive, as shown in Table 5. For these studies different antibodies detecting N-terminus [31, 36] or extracellular part of GCPII [139] or combination of both [34, 138, 142] were used. Interestingly, no GCPII positivity was detected in benign tissue [138, 142] except benign kidney [142] and also only small number of prostate cancer neovasculature showed GCPII staining (only 2 from 12) [138].

Fig. 14: Example of GCPII expression in neovasculature in clear cell carcinoma of kidney. Note that the tumor cells are negative for GCPII. Adopted from [139].

<table>
<thead>
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<th>glioblastoma multiforme</th>
<th>kidney</th>
<th>lung</th>
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<th>neuroendocrine carcinoma</th>
<th>pancreas</th>
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Table 5: GCPII expression in carcinoma neovasculature. 1, benign kidney was the only benign tissue that was GCPII positive. Different antibodies were used for detection.
The last published study detected only small samples of GCPII positive neovasculature. Tissue microarrays\(^{19}\) used in this study (described in [143]), where limited stroma is usually seen, makes the evaluation of GCPII expression in neovasculature difficult.

The expression of GCPII in neovasculature is a very interesting issue, because antibodies against GCPII can be used for delivery of a variety of agents aimed at destroying tumor neovasculature.

### 3.3.1.2.3.3 GCPII specificity and sensitivity

Sensitivity refers to the test’s ability to identify people who have the disease. Specificity refers to the test’s ability to identify people who do not have the disease. Most tumor markers are not sensitive or specific enough to be used for cancer screening.

The sensitivity and specificity of GCPII to distinguish adenocarcinoma of prostate from all other tumor types was 66% and 95%, respectively. However, in the differential diagnosis between prostate carcinoma and urothelial carcinoma of the bladder, the specificity is lower (83%) but the sensitivity is the same (66%) [139].

**EVEN THOUGH GCPII IS EXPRESSED IN SEVERAL OTHER TYPES OF MALIGNANCIES, IT REMAINS A SENSITIVE AND SPECIFIC MARKER FOR PROSTATE CARCINOMA.**

### 3.3.1.2.3.4 Simple and cost-efficient test for GCPII and cancer prognosis

#### 3.3.1.2.3.4.1 Serum screening

Testing the cancer markers directly from serum (true for PSA) would be easier way and also less expensive, but there are controversial data concerning the presence and diagnostic relevance of GCPII in human serum [144-147]. Thus GCPII is neither used as a serum diagnostic nor serum-screening marker.

\(^{19}\) Glossary item
3.3.1.2.3.4.2 Detection of GCPII mRNA

GCPII mRNA expression is not prostate specific [32, 136, 142, 148-150]. Interestingly enough, it was not detected in normal (non-tumor) vasculature [142].

The reverse transcriptase-polymerase chain reaction (RT-PCR) can be an important molecular tool for its sensitivity and specificity [151-155]. Even though the GCPII as a PCR marker has some advantage over the PSA during hormone deprivation therapy [156], GCPII RT-PCR (compared with RT-PCR of serum PSA) is still inadequate for detection of all patients with prostate carcinoma and metastasis [153, 157].

A strategy using GCPII and PSA RT-PCR assays in combination showed correlation with pathologic stage, sensitivity 67% in predicting circulating prostatic cells and specificity 91% [158-161].

RNAse protection assay showed higher expression of GCPII mRNA in prostate cancer compared to normal or benign prostate [8, 153, 162]. Also in situ hybridization studies showed trend in increasing GCPII expression with Gleason score [163]. But none of these studies did take into account existence of all alternatively spliced variants: GCPII (PSMA), PSM\(^{\prime}\), PSM-C, PSM-D and PSM-E [4, 11], which can cause false positive results.

The most relevant splice variant PSM\(^{\prime}\) (see chapter 3.1.1), lacking 266 nucleotides at the 5' end [8], represents together with GCPII a tumor index GCPII/PSM\(^{\prime}\). This index increases from normal to cancerous prostate. The ratio is also higher in the metastasis compared to normal prostate but did not change between tumor and benign tissues [7, 8].

3.3.1.2.3.4.3 Detection of GCPII protein: ProstaScint scan

GCPII protein levels are approximately 50-fold higher in normal and diseased prostate than in non-prostatic studied tissues [37]. Its expression increases from benign hyperplasia of the prostate to high-grade intraepithelial neoplasia [22] or adenocarcinoma of the prostate [164, 165-167]. GCPII expression is higher in poorly differentiated and metastatic tumors [166] and its increase correlates with tumor grade (Gleason score), biochemical recurrence, pathological stage, and in primary tumors predicts disease outcome [166-168].

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Glossary item

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20-22
In 1999 a radiographic test that uses murine antibody 7E11 linked to $^{111}$indium ($^{111}$indium-capromab pendetide = ProstaScint) was approved by US Food and Drug Administration.

ProstaScint is noninvasive scan and can differentiate patients with organ localized disease from those with metastatic prostate cancer [169, 170]. Its cost is approximately $2000 [169]. In high-risk metastatic prostate cancer this scan have demonstrated a specificity and sensitivity of 70-90% and 60-80%, respectively, which is in true better than the accuracy of current CT scans (Computerized Axial Tomography) and MRI (Magnetic Resonance Imaging) [168, 169].

An important study in 2003 showed that the ProstaScint scan does not predict biochemical control after radiation therapy [171].

### 3.3.2 Therapeutic target in the prostate cancer - GCPII again

#### 3.3.2.1 Radioimmunotherapy

In the terms of an *in vivo* targeting by radioimmunotherapy and cell killing, GCPII can be a nearly ideal molecule, because it is anchored in the membrane and is expressed in all prostate cancers [36, 156, 165]. The most used monoclonal antibody (mAb) for radioimmunotherapy is J591 stably bound to alpha-emitting radioisotope $^{213}$Bi [172-174] or to beta-emitting particles $^{111}$In, $^{131}$I, $^{90}$Y, and $^{177}$Lu [175, 176, 177-179].

Alpha-emitting and also beta-emitting mAb construct specifically targeted LNCaP cells and also prostate cancer *in vivo* [172, 174, 177] and showed no side effect [173].

Phase I radioimmunotherapy trials were started in patients with progressing hormone independent prostate cancer and J591 (labeled with $^{111}$In) targeted bone and/or soft tissue lesion in 98% of patients [176, 179]. Selective targeting of antibody to tumor was seen [175].

#### 3.3.2.2 Immunotherapeutic approach

Active non-specific immunotherapy, which uses the responses of immune system, is mostly used in cancer immunotherapy.

Dendritic cell based immunotherapy is a promising approach to increase tumor antigen-specific T-cell responses in cancer patients. This approach uses peptides from GCPII
and evokes responses of cell-mediated immunity [180-187]. T-cells were also stimulated by recombinant and native GCPII [188, 189]. In all these studies T-cells were stimulated and immune response was observed.

Since the tumor is trying to escape the immune system by down-regulation of target antigen, it seems better to generate T-cell response by several prostate-specific antigens in the same time. A study using PSCA, PAP, PSA and GCPII resulted in significant cytotoxic T-cell responses against all prostate-specific antigens tested [190].

Another interesting approach is generation of artificial T-cell receptors [191-194] or DNA vaccinations with plasmid coding for GCPII [195, 196].

Interestingly enough, prostate cancer patients immunized with plasmid coding for GCPII [197], or extracellular portion of GCPII [189, 198], or only with a part of extracellular domain [199] induced anti-GCPII humoral immune response and also produced specific antibodies which could not be detected in healthy men, women and patients before the vaccination [200].

### 3.3.2.3 Immunotoxins

Using antibodies specific to tumors conjugated with toxins is a very elegant method. Use of monoclonal antibodies to GCPII showed to be specific and effective. J591 linked to maytansine derivate (DM1, from ethiopian shrug Maytenus serrata) showed to be effectively delivered to GCPII positive cells \textit{in vitro} and \textit{in vivo} [201] and to xenograft tumor tissue. It blocked tumor growth and elaboration of osteoblastic lesions [202].

Another toxin, ricine A-chain [203] and its deglycosylated form [204] were cross-linked to different monoclonal antibodies of GCPII. The toxic effect on LNCaP cells and spheroids was observed [203]. They also inhibited growth of LNCaP tumor xenografts without toxicity in mice [204]. One of the recent attempts used recombinant immunotoxin containing single-chain antibody fragment against GCPII conjugated to truncated form of Pseudomonas exotoxin A, which binds to GCPII positive cells and reduces the viability by 50% at concentration of 20pM, while the GCPII negative cells remain unaffected [205].
3.3.2.4 Gene therapy and enhancer/promoter of GCPII

Transcription activity of GCPII enhancer/promoter is prostate specific [206]. When GCPII enhancer/promoter is combined with a cytosine deaminase gene for suicide-driven gene therapy, GCPII enhancer/promoter drives the cytosine deaminase toxicity in GCPII expressing cells and non-prostatic cells are not significantly affected [207]. Elimination of tumors when expressing cytosine deaminase under the regulatory control of GCPII enhancer/promoter was detected in mice bearing prostatic cell line transfected with enhancer/promoter of GCPII [208]. Cytosine deaminase gene suicide therapy is based on conversion of nontoxic prodrug 5-fluorocytosine into cytotoxic 5-fluorouracil. Also another suicide gene thymidine kinase, when coupled to GCPII enhancer/promoter, showed strong inhibitory effect on tumor growth in mice [209].
The principal aims of the studies presented in this thesis were:

- Analysis of possible domain representation of GCPII; the significance of these domains in protein folding and enzyme activity

- Analysis of the substrate specificity of GCPII by site-directed mutagenesis; molecular modeling; kinetic characterization

- Localization and trafficking of GCPII and its truncated form PSM' in cells and tissues
5 LIST OF PUBLICATIONS

**Mapping of the active site of glutamate carboxypeptidase II by site-directed mutagenesis.**


**Amino acids at the N- and C-termini of human glutamate carboxypeptidase II are required for enzymatic activity and proper folding.**


**Structural insight into the pharmacophore pocket of human glutamate carboxypeptidase II.**


**Biochemical characterization of human glutamate carboxypeptidase III.**


**Expression of glutamate carboxypeptidase II in human brain.**

Neuroscience 2007; 144:1361-72.

VI. Mlčochová P, Bařinka C, Konvalinka J.

**Prostate-Specific Membrane Antigen and its truncated form PSM’.**

Manuscript in preparation
Another publication (not included in thesis)


Tissue expression and enzymologic characterization of human prostate specific membrane antigen and its rat and pig orthologs.
Prostate 2007, accepted September 2007
6 RESULTS AND DISCUSSION
6.1 Importance of amino acid residues inside the active site of GCPII

6.1.1 Background information

GCPII is a metallopeptidase from the peptidase family M28. Based on the alignment of GCPII sequence to aminopeptidases Aeromonas proteolytica and Streptomyces griseus, five putative amino acids coordinating the zinc ions in the active site were identified. It has been shown that mutation in these residues (His377, Asp387, Glu425, Asp453 and His553) abolished the GCPII activity. The mutation in the putative substrate-binding residues influenced but did not abolish the GCPII activity [210]. Reliability of assignment of zinc binding residues was confirmed by the crystal structures of GCPII published in 2005 and 2006 [25, 26]. Nevertheless, the identity of substrate-binding residues is still not confirmed, because crystal structure of GCPII in complex with the naturally occurring substrate, N-acetyl-aspartyl-glutamate (NAAG), has not yet been published.

We created a computational model of GCPII/NAAG complex on the basis of crystal structure of GCPII with inhibitor GPI-18431 (Fig. 15) [26]. We found several important amino acid residues in the active site, which bind the substrate and performed the mutagenesis study to confirm this model.

![Active site of GCPII with bound inhibitor GPI-18431.](image)

Fig. 15: Active site of GCPII with bound inhibitor GPI-18431.
6.1.2 Summary

We performed detailed analysis of amino acid residues inside the active site of glutamate carboxypeptidase II using site-directed mutagenesis.

Firstly, we designed QM/MM model of GCPII/NAAG complex (GCPII in complex with N-acetyl-aspartyl-glutamate) based on known crystal structure of GCPII in complex with the inhibitor. The structural arrangement and the interactions between enzyme and substrate in S1’ pocket of GCPII/NAAG model is highly similar to crystal structure of GCPII/glutamate.

In the S1 pocket, Arg534, Arg536, and Asn519 interact with the aspartate side chain of NAAG. Mutation in these residues caused a decrease in turnover number and thus they seem to be much more important for substrate turnover than for substrate binding.

On the other hand, mutation in residues of S1’ site showed dramatic increase in Michaelis-Menten constant value (compared to wild-type). These amino acids binding glutamate of NAAG are thus critical for substrate/inhibitor recognition and binding.


**Figure 1.** Expression of PSMA/PSM’ proteins in two cell lines. **Panel A:** Lysates from LNCaP and HEK1-750 cells (untreated or deglycosylated using PNGase F) were subjected to SDS-PAGE, electroblotted on a nitrocellulose membrane, probed using the GCP-04 antibody (1:5,000). **Panel B:** Sequential immunoprecipitation of PSMA from LNCaP cells with 7E11 (against N-terminus), followed by immunoprecipitation with GCP-05 antibody (against extracellular domain). Immunoprecipitated proteins were separated on SDS-PAGE and electroblotted onto a nitrocellulose membrane, then probed with GCP-04 antibody.

**Figure 2.** Effect of endoglycosidases on PSMA/PSM’ proteins. Lysates from LNCaP (Panel A) and HEK1-750 cells (Panel B) were either untreated or deglycosylated using PNGase F or Endo H. Protein samples were resolved by SDS-PAGE, electroblotted on a nitrocellulose membrane, and the membrane was probed with the GCP-04 antibody (1:5,000). Panels C, D: PSMA/PSM’ from LNCaP (Panel C) and HEK1-750 (Panel D) lysates were sequentially immunoprecipitated using monoclonal antibodies 7E11 and GCP-05, and proteins were subsequently deglycosylated with PNGase F or Endo H. Samples were resolved by SDS-PAGE, electroblotted on a nitrocellulose membrane, and the membrane was probed with the GCP-04 antibody (1:5,000)

**Figure 3.** PSM’ localization to cytosol. Lysates from LNCaP and HEK1-750 cells were subjected to differential centrifugation and individual fractions were inspected for the presence of PSMA/PSM’ proteins (Western blotting using the GCP-04 antibody; Panels A, B) and the NAAG-hydrolyzing activity (Panels C, D). Panel A: Immunodetection of PSMA/PSM’ in different cellular fractions. Panel B: Immunodetection of untreated or PNGase F treated macromolecule (Ma) and cytosolic (Cyt) fractions. Panels C, D: Individual fractions from the differential centrifugation of LNCaP (Panel C) and HEK1-750 (Panel D) lysates were assayed for the NAAG-hydrolyzing activity using the radioenzymatic assay.

**Figure 4.** Cell surface biotinylation. Cell surface proteins were biotinylated with Sulfo-NHS-SS-Biotin and then incubated 1h at 37°C to allow internalization of plasma membrane molecules. Biotin labels were stripped from proteins remaining at the cell surface, cells were lysed and cell lysates subjected to sequential immunoprecipitation using monoclonal antibodies 7E11 (recognizing the N-terminus) and GCP-05 (to the extracellular domain). Proteins were resolved by SDS-PAGE and electroblotted on a nitrocellulose membrane. Panel A: The immunoblot probed with the GCP-04 antibody (1:5,000). Panel B: The immunoblot probed with NeutrAvidin conjugated to horseradish peroxidase (1:2,500) specific for biotin.
Figure 1

![Figure 1](image)

Figure 2

![Figure 2](image)
Figure 3

A. Western blot analysis showing expression of PSMA and PSM in LNCaP and HEK 1-750 cell lines.

B. Gelatin zymography showing gelatinase activity in LNCaP and HEK 1-750.

C. Enzyme activity assay showing activity in LNCaP lysates.

D. Enzyme activity assay showing activity in HEK 1-750 lysates.

Figure 4

A. Western blot analysis showing PSMA and PSM expression in TEs11 and GCP-05 cell lines.

B. Western blot analysis showing PSMA expression in GCP-04 and NA-HRP.
7 CONCLUSIONS AND PERSPECTIVES

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Recently reported crystal structures of GCPII provide structural insight into the organization of the substrate binding cavity and highlight residues implicated in substrate/inhibitor binding in the S1’ site of the enzyme. To complement and extend the structural studies, we constructed a QM/MM model of GCPII in complex with its substrate, N-acetyl-aspartyl-glutamate, which enabled us to predict additional amino acid residues interacting with the bound substrate, and used site-directed mutagenesis to assess the contribution of individual residues for substrate/inhibitor binding and enzymatic activity of GCPII. We prepared and characterized 12 GCPII mutants targeting the amino acids in the vicinity of substrate/inhibitor binding pockets. The experimental results suggest that residues (especially Arg210) in the S1’ site are critical for substrate/inhibitor binding, whereas the residues forming the S1 pocket might be more important for the ‘fine-tuning’ of GCPII substrate specificity and appear to be relevant for substrate turnover and may play a role in the enzyme’s mechanism of action.

Even though the QM/MM calculations of the NAAG binding mode in the GCPII active site enabled us to predict the structure and enzyme–substrate interactions in the S1 binding site, the complete description of reaction mechanism of GCPII is beyond the scope of our study. We would like to look more closely into the catalytic mechanism of glutamate carboxypeptidase II. An interesting approach is a study of a putative proton shuttle Glu424, located near zinc ions in the active site of GCPII. The mutation of this residue might show us its role in enzyme catalysis; moreover, we might obtain a crystal structure of GCPII with bound unsplit substrate N-acetyl-aspartyl-glutamate.

Human GCPII consists of 750 amino acids, and six individual domains were predicted to constitute the protein structure. We reported the analysis of the contribution of these putative domains to the structure and function of recombinant human GCPII. We cloned 13 mutants of human GCPII that are truncated or extended at one or both the N- and C-termini of the GCPII sequence. The clones were used to generate stably transfected Drosophila Schneider’s cells, and the expression and carboxypeptidase activities of the individual protein products were determined. The results clearly show that the amino acids at the extreme C-terminus of GCPII are crucial for the hydrolytic activity of the enzyme and, furthermore, that no more than 60 amino acids can be deleted from the N-terminus without compromising the carboxypeptidase activity of GCPII.

We undertook this study before the first crystal structure of GCPII was determined. X-ray structures provided evidence that the ectodomain of GCPII is composed of three domains.
All these domains form active site of the enzyme and are indispensable for the GCPII enzymatic activity, which explains why changes on both N- and C- terminus are so detrimental to protein stability and activity.

We report crystal structures of the human GCPII complexed with three glutamate mimetics/derivatives, 2-(phosphonomethyl)pentanedioic acid, quisqualic acid, and L-serine $O$-sulfate. Despite the structural differences between the distal parts of the inhibitors, all three compounds share similar binding modes in the S1’ site (pharmacophore pocket) of GCPII, where they are stabilized by a combination of polar and van der Waals interactions. The structural variety of the distal parts of the inhibitors leads to rearrangements of the S1’ site that are necessary for efficient interactions between the enzyme and an inhibitor.

The set of structures presented here, in connection with the available biochemical data, illustrates a flexibility of the GCPII pharmacophore pocket and underlines the structural features required for potent GCPII inhibition.

Our data could be used for the development of the new GCPII inhibitors using the rational structure-based drug design approach and could draw attention to the modification in the inhibitor structure, which can improve the pharmacokinetic profile and potency towards GCPII.

Experiments with GCPII knock-out mice showed that GCPII is not the only one NAAG-hydrolyzing enzyme in the brain. We presumed that glutamate carboxypeptidase III (GCPIII), a close homolog of GCPII, might complement for GCPII activity in these knock-out mice.

While human GCPII is an important pharmacological target in the neurotransmission and degenerative diseases, no biochemical study of human GCPIII is available at present. We cloned, expressed and characterized a recombinant human GCPIII.

We show that GCPIII lacks dipeptidylpeptidase IV-like activity, its activity is dependent on N-glycosylation, and is sensitive to several known inhibitors of GCPII effectively inhibit it. In comparison to GCPII, GCPIII has lower N-acetyl-aspartyl-glutamate-hydrolyzing activity, different pH and salt concentration dependence, and distinct substrate specificity.

We created a molecular model of GCPIII and provided interpretation of the distinct substrate specificity of both enzymes, and examine the amino acid residues responsible for the differences by site-directed mutagenesis. These results may help to design potent and
selective inhibitors of both enzymes. Such inhibitors would be helpful to evaluate and distinguish biological roles of the two individual enzymes.

We believe that GCPIII activity is significant enough to account for the NAAG-hydrolyzing activity observed in the tissues of GCPII knock-out mice and that GCPIII might thus represent a valid pharmaceutical target.

Why would brain harbour two similar enzymes with the same enzymatic activity? The honest answer is: we do not know. The possible explanation might be that GCPII and GCPIII possess different biological roles in the brain. One of our goals is to find GCPIII molecular partner and to clarify the function of GCPIII.

Only very limited and controversial data on the expression and localization of GCPII in human brain are available. Therefore, we set out the first systematic analysis of the expression of GCPII in human brain using immunochemical detection. We used a novel monoclonal antibody GCP-04, which recognizes an epitope on the extracellular part of the enzyme and is more sensitive to GCPII than to the homologous protein GCPIII. We also showed that this antibody is more sensitive in immunoblots than the widely used antibody 7E11. Immunohistochemical analysis revealed GCPII expression in all parts of the human brain. GCPII seems to be expressed exclusively in astrocytes, especially in those localized in the white matter. Our published results are only starting point in further studies on the role of GCPII in the human brain.

It is generally known that GCPII is expressed in prostate and overexpressed during the prostate cancer. Analogically, we showed GCPII expression in astrocytes and we would like to investigate further the GCPII expression in brain tumors, especially in the astrocytomas.

In the benign prostate PSM’ mRNA is overexpressed over GCPII. Interestingly enough, in the case of prostate cancer this expression pattern is reversed. Very few information is known about protein designated PSM’, a truncated form of GCPII. We investigated the origin of PSM’ and its trafficking in the cells.

Our experiments revealed that PSM’ is a proteolytically active N-linked glycoprotein. Surprisingly, it is not a product of alternatively spliced GCPII mRNA, which is generally accepted fact. We hypothesize that it might be a product of proteolytic processing of the full length GCPII upon internalization and endosomal trafficking, but our data suggest it is also not the case. We can only speculate that this species might be produced by a proteolytic
processing event inside the Golgi apparatus and then translocated by an unknown mechanism into the cytosol.

Insights into GCPII processing and the origin of its truncated form PSM’ might improve our understanding of the behavior of GCPII, a therapeutic target for prostate cancer, as well as our general understanding of N-glycosylation and the trafficking of cytosolic proteins.
8 REFERENCES

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activity and some stereotypes in the PCP model of schizophrenia via group II mGluR. J Neurochem 2004;89:876-885.


**1 FISH**
Fluorescent *in situ* hybridization is a cytogenetic technique, which is used for detection and localization of presence or absence of specific DNA sequences on chromosomes. It uses fluorescent probes, which bind only to those parts of the chromosome with which they show a high degree of sequence similarity. Fluorescence microscopy can be used to find out where the fluorescent probe bound to the chromosome.

**2 agonist**
In pharmacology it is a substance that binds to a specific receptor and triggers a response in the cell. It mimics the action of an endogenous ligand (such as hormone or neurotransmitter) that binds to the same receptor.

**3 G-proteins**
Guanine nucleotide binding proteins are a family of proteins involved in second messenger cascades. They are so called, because of their signaling mechanism, which uses the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) as a general molecular "switch" function to regulate cell processes.

**4 antagonist**
A molecule that blocks the ability of a given chemical to bind to its receptor, it prevents a biological response.

**5 glia (glial cells)**
Non-neuronal cells that provide support and nutrition, maintain homeostasis, form myelin, and participate in signal transmission in the nervous system.

*Types of glia:*

**Microglia**
Specialized macrophages capable of phagocytosis that protect neurons of the central nervous system.

**Astrocytes**
Characteristic star-shaped glial cells in the brain. They perform many functions, including the formation of the blood-brain barrier, the provision of nutrients to the nervous tissue, and play a principal role in the repair and scarring process in the brain.

**Oligodendrocytes**
Cells that coat axons in the central nervous system with their cell membrane, called myelin, producing the so-called myelin sheath.

**Ependymal cells**
Cell which line the cavities of the CNS and make up the walls of the ventricles. These cells create and secrete cerebrospinal fluid.

**Schwann cells**
They are similar in function to oligodendrocytes and provide myelination to axons in the peripheral nervous system. They also have phagocytotic activity.

**6 transient middle cerebral artery occlusion**
It is an animal model of ischemic stroke where the middle cerebral artery is surgically dissected and subsequently transiently occluded. After defined period of time the middle cerebral artery is being reperfused again.
7 **ganglion**
Type of neuron located in the retina of the eye that receives visual information from photoreceptors via various intermediate cells such as bipolar cells, amacrine cells, and horizontal cells.

8 **traumatic brain injury**
Traumatic injuries to the brain occur when a sudden trauma causes brain damage. Symptoms of a traumatic brain injury can be mild, moderate, or severe, depending on the extent of the damage to the brain. Outcome can be anything from complete recovery to permanent disability or death.

9 **lateral fluid percussion**
A model of mild-moderate concussion, which leads to the temporary loss of the capacity for experience-dependent plasticity in developing organism.

10 **afferent nerves**
Nerve fibers (usually sensory) that carry impulses from an organ or tissue toward the brain and spinal cord, or the information processing centers of the enteric nervous system, which is located within the walls of the digestive tract.

11 **ectopic discharges**
The ectopic afferent activity is largely responsible for the development of hypersensitivity of dorsal horn neurons and neuropathic pain. Afferent ectopic discharges from the site of nerve injury constitute a source of abnormal sensory input to the spinal dorsal horn.

12 **hyperalgesia**
An extreme sensitivity to pain, which in one form is caused by damage to nociceptors in the body's soft tissues.
A nociceptor is a sensory receptor that sends signals that cause the perception of pain in response to potentially damaging stimulus.

13 **allodynia**
An exaggerated response to otherwise non-noxious stimuli and can be either static or mechanical. For example, a person with allodynia may perceive light pressure or the movement of clothes over the skin as painful, whereas a healthy individual will not feel pain.

14 **carrageenan injection**
In this method carrageenan is injected subcutaneously into the plantar surface of the hind paw.

15 **diabetic neuropathy**
Disorders associated with diabetes mellitus, which result from diabetic microvascular injury involving small blood vessels that supply nerves. The first pathological change in the microvasculature is vasoconstriction. As the disease progresses, neuronal dysfunction correlates closely with the development of vascular abnormalities, which contribute to diminished oxygen tension and hypoxia. Microvascular dysfunction occurs early in diabetes, parallels the progression of neural dysfunction, and may be sufficient to support the severity of structural, functional, and clinical changes observed in diabetic neuropathy.

16 **carcinoma**
Any cancer that arises from epithelial cells. It is malignant by definition: carcinomas invade surrounding tissues and organs, and may spread to lymph nodes and distal sites (metastasis).

17 **benign prostatic hyperplasia (BPH)**
Increase in size of the prostate in middle-aged and elderly men. It is characterized by hyperplasia of prostatic stromal and epithelial cells, resulting in the formation of large, fairly discrete nodules in the
periurethral region of the prostate. Although prostate specific antigen levels may be elevated in these patients, because of increased organ volume and inflammation due to urinary tract infections, BPH is not considered to be a premalignant lesion.

18 **adenocarcinoma**
Form of carcinoma that originates in glandular tissue. To be classified as adenocarcinoma, the cells do not necessarily need to be part of a gland, as long as they have secretory properties. This form of carcinoma can occur in some higher mammals, including humans.

19 **tissue microarrays**
A paraffin blocks in which up to 1000 separate tissue cores are assembled in array fashion to allow simultaneous histological analysis. The major limitations in molecular clinical analysis of tissues include the cumbersome nature of procedures, limited availability of diagnostic reagents and limited patient sample size. The technique of tissue microarray was developed to address these issues.

20 **metastasis**
Spread of a disease from one organ or part to another non-contiguous organ or part. Only malignant tumor cells and infections have the capacity to metastasize.

21 **Gleason score**
A Gleason score is given to prostate cancer based upon its microscopic appearance. The Gleason score is important because higher Gleason scores are associated with worse prognosis. This is because higher Gleason scores are given to cancer, which is more aggressive. To assign a Gleason score, a piece of prostatic tissue must be obtained (a biopsy). This is done either by removing the gland (prostatectomy) or by sampling the gland with a needle introduced through the rectum.

- **Grade 1**: The cancerous prostate closely resembles normal prostate tissue. The glands are small, well formed, and closely packed
- **Grade 2**: The tissue still has well-formed glands, but they are larger and have more tissue between them.
- **Grade 3**: The tissue still has recognizable glands, but the cells are darker. At high magnification, some of these cells have left the glands and are beginning to invade the surrounding tissue.
- **Grade 4**: The tissue has few recognizable glands. Many cells are invading the surrounding tissue
- **Grade 5**: The tissue does not have recognizable glands. There are often just sheets of cells throughout the surrounding tissue.

22 **prostatic intraepithelial neoplasia (PIN)**
A non-invasive lesion in the prostate gland that is thought to be a precursor to prostate cancer. PIN does not require specific therapy, but close follow-up with additional biopsies is warranted. PIN may disappear, remain unchanged, or progress to prostate cancer, often over as many as ten years.