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Glutamate Carboxypeptidase II in Diagnosis and Treatment of Neurologic Disorders and Prostate Cancer

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Abstract

Glutamate carboxypeptidase II (GCPII) is a membrane-bound binuclear zinc metallopeptidase with the highest expression levels found in the nervous and prostatic tissue. Throughout the nervous system, glia-bound GCPII is intimately involved in the neuron-neuron and neuron-glia signaling *via* the hydrolysis of N-acetylaspartylglutamate (NAAG), the most abundant mammalian peptidic neurotransmitter. The inhibition of the GCPII-controlled NAAG catabolism has been shown to attenuate neurotoxicity associated with enhanced glutamate transmission and GCPII-specific inhibitors demonstrate efficacy in multiple preclinical models including traumatic brain injury, stroke, neuropathic and inflammatory pain, amyotrophic lateral sclerosis, and schizophrenia. The second major area of pharmacological interventions targeting GCPII focuses on prostate carcinoma; GCPII expression levels are highly increased in androgen-independent and metastatic disease. Consequently, the enzyme serves as a potential target for imaging and therapy. This review offers a summary of GCPII structure, physiological functions in healthy tissues, and its association with various pathologies. The review also outlines the development of GCPII-specific small-molecule compounds and their use in preclinical and clinical settings.

Keywords

Metalloprotease; prostate-specific membrane antigen; glutamate excitotoxicity; prostate cancer; N-acetylaspartylglutamate

1. INTRODUCTION

Glutamate carboxypetidase II (GCPII; EC 3.4.17.21) also known as prostate specific membrane antigen (PSMA), N-acetylated-alpha-linked acidic dipeptidase (NAALADase), and folate hydrolase (FOLH1) is a zinc-dependent peptidase that is increasingly recognized as a target of therapeutic interventions in a variety of neurologic disorders as well as a marker for imaging of and therapy for prostate cancer (PCa). GCPII was identified by different laboratories in different tissues of different species more than 20 years ago; original designations reflected the belief that the identified protein was either a unique entity in a

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given organ (PSMA) or had a single defined role (NAALADase). Even though original designations still persist in the literature, the term glutamate carboxypeptidase II (GCPII) is now preferred and will be used throughout this review.

2. GCPII STRUCTURE

2.1. Primary Structure

Human GCPII conforms to the pattern typical for type II transmembrane proteins having a short N-terminal cytoplasmic tail (amino acids 1 – 18), a single membrane-spanning helix (amino acids 19 – 43) and a large extracellular part (amino acids 44 – 750).

The N-terminal GCPII tail interacts with several scaffold proteins including clathrin, clathrin adaptor protein 2, filamin A, and caveolin-1. These interactions govern/modulate GCPII endocytosis *via* different routes, including caveolae-dependent and clathrin-coated pit-dependent mechanisms [1–4]. In the case of clathrin-dependent trafficking, the MXXXL N-terminal motif is indispensable for GCPII internalization and recycling [4]. GCPII is internalized in a constitutive manner, yet the internalization rate is increased by the binding of GCPII-specific antibodies to the extracellular domain of the protein [5]. These findings are being exploited for the development of therapeutic approaches to target the delivery of toxins, drugs, and short-range isotopes to the interior of GCPII-expressing cells.

The bulk of the protein is oriented to the extracellular milieu, where it can act on its natural substrates (Fig. 1; see Chapter 3.3). The extracellular portion of GCPII homodimerizes and the dimerization is believed to be required for GCPII hydrolytic activity [6], even though the active site in each subunit is structurally independent [7]. GCPII is also heavily N- and O-glycosylated (glycans can account for up to 25% of the total molecular weight of the protein); there are ten N-glycosylation sites predicted within the primary sequence of human GCPII and the N-glycosylation is indispensable for GCPII enzymatic activity and stability [8–12]. Furthermore, glycosylation of the protein is implicated in apical sorting, proteolytic resistance and its association with lipid rafts [13,14].

2.2. Tertiary Structure

The 3-dimensional structure of the human GCPII ectodomain was solved by two groups independently [7, 15]. The overall fold closely resembles the structure of the transferrin receptor [16]. The extracellular part of GCPII consists of three distinct domains spanning amino acids 57–116 and 352–590 (the protease domain), 117–351 (the apical domain), and 591–750 (the C-terminal or dimerization domain). Synergetic action of all three domains is required for productive substrate binding and processing, as several residues from each domain contribute to the architecture of the GCPII substrate binding cavity and are involved in ligand recognition [7].

The GCPII substrate binding cavity is divided by the active site (featuring two zinc ions) into two “halves”, designated the S1’ pocket and the S1 pocket, respectively. The binuclear zinc active site, with the two zinc ions coordinated by the side chains of His377, Asp387, Glu425, Asp453, and His553, is indispensable for the GCPII hydrolytic activity [17,18]. It is also exploited for the design of high-affinity inhibitors as every high-affinity GCPII inhibitor includes a zinc-binding group in its structure. Amino acid residues shaping S1 and S1’ pockets dictate GCPII preferences towards physicochemical characteristics of cognate substrates and small-molecule inhibitors.

The S1’ pocket, also termed a pharmacophore pocket, is ‘optimized’ for binding of glutamate and glutamate-like moieties [19–21]. Not surprisingly then, both known natural GCPII substrates (NAAG and folyl-poly-γ-glutamates) feature glutamate as the C-terminal

residue. Additionally, the majority of GCPII-specific inhibitors are derived from glutamate (or NAAG) scaffolds to take the advantage of S1' pocket affinity towards glutamate. It should be noted, however, that although glutamate-like moieties are preferred in the S1' pocket, the pocket has an amphipathic character, i.e. exploits both polar (hydrogen-bonding, ionic) and non-polar (hydrophobic, van der Waals) interactions to accommodate and stabilize binding of substrate or inhibitor [8,19,22]. Consequently, the glutamate-like functionality can be substituted by a more hydrophobic moiety if an inhibitor is to be used in settings, where decreased polarity is desired (such as brain-penetrating compounds) [21, 23].

The S1 pocket of GCPII is fairly specific for glutamate and aspartate side chains, both negatively charged amino acids. This specificity is conferred by the "arginine patch", a conspicuous spatial arrangement of Arg534, Arg536 and Arg463 side chains [24]. Even though GCPII selectivity for negatively charged residues in the S1 pocket is virtually absolute in the case of substrates, a wide variety of corresponding distal parts (D-parts) of inhibitors is tolerated here. In contrast to a small (approximate dimensions 8 × 8 × 8 Å) and unyielding S1' pocket [19], the "extended" S1 pocket can be described as a shape-shifting funnel with a narrow base (~8 Å) at the position of the active-site zinc ions, and the rim of the funnel (an approximate diameter of 20 Å) at a distance 20 Å from the base. Large dimensions of the S1 funnel thus allow for considerable variability of the D-moiety in the inhibitor; these characteristics have been extensively used in inhibitor design [25–31].

3. GCPII IN PHYSIOLOGY

3.1. GCPII Localization

In 1987, the world was simple and straightforward. A newly developed 7E11 antibody (see Chapter 4.2. for its current clinical use) recognizing the N-terminal part of GCPII localized GCPII expression solely to the prostatic tissue (consequently termed prostate specific membrane antigen – PSMA) [32]. Later, however, PSMA was shown to be identical to GCPII or NAALADase as it was termed then [33]. With the development of novel, more sensitive antibodies and more data available, the picture of GCPII expression pattern became more complex [34–40].

Today in healthy human tissue a consensus has been reached for the following sites of GCPII expression: prostate (secretory-acinar epithelium) [35,38–41], nervous system (astrocytes and schwann cells) [42,37], kidney (proximal tubules) [35,36,38,43,44], and small intestine (jejunal brush border membranes) [35,36,38–40]. However, several other studies have pointed towards a broader GCPII expression profile in humans, including expression in heart, pancreas, bladder, skin, breast, liver, lung, colon, testis, etc., although the expression levels in the latter tissues is thought to be much lower compared to "consensus sites" [34,36,39]. To complicate things further, *in vitro* data (immunoblots, immunohistochemistry) mentioned above do not offer the same picture as *in vivo* data using GCPII ligands (small molecules and/or labeled antibodies) for imaging studies. In *in vivo* studies using humans and/or laboratory animals, off-target binding is typically absent, suggesting restricted GCPII expression [45–49]. The underlying reason for these differences is not known at present, though it might be associated with the restricted accessibility of GCPII for small molecules/antibodies *in vivo* compared to *in vitro* studies or the epitopes/binding sites being masked by posttranslational modifications or putative interaction partners.

From all malignant tissues, the highest levels of GCPII are found in androgen resistant prostate cancer, with expression levels increasing with increasing cancer grade [41]. Not surprisingly, prostate cancer is the prime target for imaging/therapy by modalities aimed at GCPII (Chapter 4.2.). In addition to prostate cancer, GCPII expression has been noticed in

malignancies from kidney, bladder, breast, colon, and schwann cells [34,35,50–52]. Highly promising for imaging/treatment of diverse solid cancers is the fact that GCPII is expressed in neovasculature of solid tumors, but absent from normal vasculature [43,51,53–55]. It remains to be seen whether this phenomenon will be exploited in the clinic.

Upregulated GCPII expression in aggressive tumors implies a role of GCPII in cancer progression. Consistent with this notion, several reports and patent applications [56,57] found strong correlation between GCPII hydrolytic activity and tumor cell growth/invasion. Yao *et al* reported that GCPII expression increases folate uptake and gives GCPII expressing cells a proliferative advantage [58,59]. Similarly, Conway *et al* found that the enzymatic activity of GCPII was required for endothelial cell invasion [60], while experiments with murine transgenic prostate tissue suggested that GCPII expression facilitates prostate carcinogenesis [61]. Contrary to these data, however, Ghosh *et al* found an inverse correlation between GCPII expression and prostate cancer cell invasiveness [62]. It remains to be rigorously tested in more experimental settings, whether or not the manipulation/inhibition of GCPII enzymatic activity can be beneficial in terms of blocking the invasiveness of prostate cancer or tumor growth.

It should be noted that there are marked differences between humans and rodents that are used as experimental models of human diseases. For example, GCPII is absent from rodent prostatic tissue [36,63] and small intestine [36,64], even though these are major sites of GCPII expression in humans. Additionally, there are no reports of GCPII expression in neovasculature of solid tumors in rodents as compared to human subjects and there are marked differences in binding of small-molecule GCPII ligands to plasma proteins between these species (Pomper, unpublished). All of these differences have to be carefully considered in the course of the development of GCPII targeting modalities.

In conclusion, more detailed studies are needed to clarify data available in the literature. First, detailed characterization of anti-GCPII antibodies is needed, including specificity (GCPII *vs.* GCPIII), sensitivity and cross-reactivity with non-human GCPII orthologs. Additionally, ultrastructural studies are urgently needed to map precisely GCPII expression to individual cell populations (and subcellular compartments) in healthy organs.

3.2. GCPII Splice Variants, Paralogs, and Related Peptidases

Pertinent to physiological functions of GCPII as well as the development of ligands/antibodies targeting the enzyme can be the existence of several GCPII paralogs (GCPIII, NAALADAse L, NAALADAse L2, PSMAL), splice variants (PSM', PSM-C – PSM-F), and M28 family members (e.g. plasma glutamate carboxypeptidase).

The best studied GCPII splice variant, designated PSM', has been first described by Su *et al* and the authors suggested that the relative ratio of GCPII/PSM' mRNA in prostate can be used for determining the "tumor index" of the prostatic tissue [65], even though the clinical significance of these findings was later questioned by others [66]. Uncertain is also the existence of the PSM' protein that would be missing the first 57 amino acids of the full length GCPII, i.e. the intracellular and transmembrane segments, and located in the cytosol of PSM' expressing cells. Although a similar protein (lacking first 59 amino acids) was indeed later isolated by Grauer *et al* [67] from the LNCaP cell line, more recent data points towards PSM' being a product of GCPII endoproteolysis rather that a transcript of the PSM' splice variant [68]. There is limited information on other GCPII splice variants at the mRNA level, and nothing is known about the existence and function of corresponding protein products.

Out of four GCPII paralogs in humans (NAALADase L, NAALADase L2, PSMAL, and GCPIII), GCPIII is the most relevant when considering the design of GCPII ligands for the treatment of disease. Human GCPIII is a type II integral membrane metallopeptidase comprising 740 amino acids and sharing 67% identity with GCPII at the amino acid level. Human GCPIII was cloned in 1999 (the mouse GCPIII ortholog in 2004) and the expression profile mapping showed mRNA presence predominantly in ovary, testes, spleen, heart, placenta and brain [69,70]. However, the physiological role of GCPIII and protein localization in humans/mice are unknown due to the lack of GCPIII-specific antibodies/ligands. Published studies using both transfected cells and purified proteins show that GCPIII hydrolyzes NAAG with kinetic parameters similar to GCPII [22,71]. Additionally, there is a significant overlap (near identity) in the substrate specificity as well as the pharmacologic profile between the two proteins. These biochemical findings were recently corroborated and rationalized by solving the GCPIII crystal structure [72]. Given the close sequence/structural similarity between GCPII and GCPIII, small-molecule ligands aimed at GCPII are very likely to interact with GCPIII with similar potency. Considering our lack of knowledge of GCPIII physiology/expression pattern, the impact of such cross-reactivity towards the intended use of GCPII-specific compounds is difficult to predict at present. Clearly, such cross-reactivity can be advantageous (e.g. the complete inhibition of NAAG-hydrolyzing activity in brain) in some settings, while undesired in other (e.g. specific GCPII targeting on cancer cells). The development of GCPIII-specific modalities (antibodies, inhibitors) and establishing GCPIII-knockout mice would definitely help in dissecting diverse physiological roles of the two proteins and be beneficial in rationalizing the design of candidate drugs targeting GCPII/GCPIII.

Plasma glutamate carboxypeptidase (PGCP) is a 56 kDa glycoprotein found in high amounts in human plasma. Both GCPII and PGCP belong to the M28 family, share 27% identity at the amino acid level and require zinc ions for proteolytic activity. Although the pharmacological profile as well as substrate specificity of human PGCP differ from GCPII, both enzymes are able to hydrolyze NAAG suggesting some structural/functional overlap between the two [73,74]. In this respect it is interesting to note that in the recent phase I clinical trial aimed at PCa imaging using *N*-[*N*-[(*S*)-1,3-dicarboxypropyl]carbamoyl]-4-[^{18}F]fluoro-benzyl-L-Cysteine ([^{18}F]DCFBC), a GCPII specific imaging agent [49], an increased signal from the blood pool was observed in human subjects, suggesting potential binding of the compound to an unidentified plasma protein. Ongoing studies shall answer the question whether this observation can be linked to the presence of PGCP or other proteins in human blood.

3.3. GCPII Physiological Functions

Although high expression levels of GCPII in advanced prostate cancer serve as an excellent target for PCa imaging/therapy, its physiological function(s) in this tissue is not known. Similarly, despite substantial expression levels of GCPII in kidneys and (human) neovasculature its physiological role remains to be defined. As a result, only two roles, both requiring GCPII enzymatic activity, are well defined at present – folate-hydrolyzing activity [75,76] and NAAG-hydrolyzing activity [77] in the small intestine and the nervous system, respectively (Fig. 1). In addition to functioning as a hydrolase/peptidase, several reports allude to a non-proteolytic role(s) of GCPII in human physiology. GCPII has been shown to facilitate integrin signaling in epithelial cells [60], to be associated with the anaphase-promoting complex in prostate cancer cells [78] and to activate the NF-κB signaling pathway promoting cell proliferation [79]. However, further studies are required to shed more light on non-enzymatic GCPII functions, including the exact mechanism(s) of transmembrane signaling and the identification of putative physiological interaction partners (if they exist).

3.3.1. GCPII as Folate Hydrolase—Dietary folates consist of a mixture of poly-γ-glutamylated species, but only mono-glutamylated compounds are actively transported through the intestinal wall into the bloodstream [80]. In humans (and pigs), trimming of glutamate tails of dietary folates is performed by GCPII located in the brush border of proximal jejunum – the folate hydrolase activity of GCPII thus plays an important role in folate absorption in humans [81]. It is interesting to note that due to the absence of GCPII expression in rat intestine, dietary folates are hydrolyzed by γ-glutamyl hydrolase, a hydrolase found in pancreatic secretions in rat [64]. The physiological significance (if any) of this difference is not known.

Folate metabolism has been reported to be influenced by polymorphism in the GCPII gene, though existing epidemiologic data are somewhat contradictory. Devlin *et al* reported 50% decreased activity of the H475Y GCPII naturally occurring variant (corresponding to the C1561T at the nucleotide level) towards folate-poly-γ-glutamates [82]. Several groups have analyzed the influence of GCPII polymorphism on folate/homocysteine concentrations and their association with the incidence of various disorders (neural tube defects, gastric cancer, prostate cancer, cardiovascular disease) [83–88]. So far, unfortunately, the epidemiologic data provide conflicting results that are difficult to reconcile. Clearly, further studies are needed to draw a more detailed picture of GCPII involvement in folate metabolism.

3.3.2. GCPII as NAAG-Hydrolase—N-acetyl-aspartyl-glutamate (NAAG) is the most prevalent and widely distributed peptidic transmitter in the mammalian brain (reaching millimolar concentrations) and its expression pattern overlaps with nearly all major neurotransmitters including glutamate, GABA, acetylcholine, dopamine and serotonin [89,90]. NAAG is synthesized in neurons from glutamate and N-acetyl-aspartate by recently discovered NAAG-synthetase I (Rimkl B) and is stored in synaptic vesicles of presynaptic axon terminals [91]. Upon depolarization, the dipeptide is released in a calcium dependent manner and rapidly diffuses from the synaptic cleft into an extrasynaptic space where it undergoes two different fates [92,93].

Intact NAAG acts as an agonist at the metabotropic glutamate receptor 3 (mGluR3) located at both presynaptic nerve terminals as well as on astrocytes apposed to the synapse. Upon mGluR3 activation, an associated G-protein coupled pathway leads to the decrease in cAMP (and cGMP) second messenger concentrations within the cell. In the presynaptic nerve terminal this action results in the decrease in the amount of glutamate that would be released upon further nerve stimulation, thus dampening the amount of the discharged neurotransmitter [94–96]. In astrocytes mGluR3 activation stimulates production and secretion of transforming growth factor β [97,98]. Both these actions of NAAG can be viewed as neuroprotective. It is therefore not surprising that mGluR3 agonists, such as LY379268, have been found to be neuroprotective in a variety of animal models of neurological conditions [99].

The catabolic arm of the NAAG fate in the extrasynaptic space relies on the presence of GCPII (and possibly GCPIII, though the role of this enzyme in NAAG metabolism is not well defined at present) at the plasma membrane of astrocytes and schwann cells in the CNS and PNS, respectively [37,42]. Here, extrasynaptic NAAG is hydrolyzed into Ac-Asp and glutamate and the reaction products are transported into astrocytes/oligodendrocytes [77,100,101]. The NAAG-hydrolyzing activity of GCPII “inactivates” NAAG, indirectly blocking its effects on mGluR3.

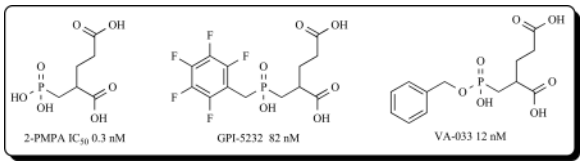
Taken into account processes described above, the following rationale is used for the development of GCPII-specific inhibitors for the treatment of neurologic conditions associated with glutamate excitotoxicity: by inhibiting the NAAG-hydrolyzing activity of

GCPII, there is a marked increase of intact NAAG (and the decrease in free glutamate as the reaction product) in the extrasynaptic space leading to the activation of mGluR3 receptors. The mGluR3 activation/signaling results in both the secretion of neuroprotective peptides by astrocytes and the reduction in glutamate release from presynaptic nerve terminals. Lower glutamate concentrations in the synaptic cleft (or extracellular milieu) are beneficial by not hyperactivating ionotropic glutamate channels that would be otherwise associated with serious pathological consequences (chapter 4.1.1.). This rationale has been extensively tested and confirmed in a variety of animal models of PNS and CNS disorders (Chapter 4.1.3.).

4. GCPII AS A THERAPEUTIC TARGET

4.1. CNS and PNS Disorders

4.1.1. Glutamate Excitotoxicity—Excessive glutamate causes neuronal dysfunction and degeneration, a phenomenon known as “glutamate excitotoxicity”. Glutamate is the most prevalent excitatory neurotransmitter in the mammalian nervous system and glutamate excitotoxicity has been inextricably linked to both acute and chronic neuronal disorders, including stroke, traumatic brain injury, amyotrophic lateral sclerosis, Alzheimer’s dementia and Parkinson’s disease. In a simplified view, the mechanisms underlying glutamate excitotoxicity are initiated by non-physiological increase in synaptic glutamate concentrations resulting in hyperactivation of ionotropic glutamate channels, the most notably N-methyl-D-aspartate receptors (NMDAR). Excessive calcium influx *via* NMDARs triggers a complex and intertwined array of cellular responses, including nitric oxide overproduction, mitochondrial dysfunction associated with free radical generation, caspase activation, or ionic homeostasis imbalance, that ultimately lead to necrotic or apoptotic death of affected neurons (reviewed in ref. [102,103]).



Pharmacologic interventions aimed at attenuating neuronal damage associated with glutamate excitotoxicity mostly concentrate on modifying/blocking downstream effector processes, and utilize ionotropic receptor antagonists, NO synthase inhibitors, free radical scavengers, caspase inhibitors etc. Unfortunately, despite the continued research in this field, there are no pharmacological interventions providing significant neuroprotection at present [102,103].

Given the failure of several NMDAR antagonists, alternative strategies focus on targeting processes upstream of the excitotoxic insult, i.e. blocking glutamate release from presynaptic terminals. The rationale of this approach is that by diminishing the amount of glutamate in the synaptic cleft, it is possible block several diverse downstream pathologic processes at once. Lamotrigin and riluzole are two examples of such strategy. Both drugs are presynaptic sodium channel blockers used in clinical practice to alleviate symptoms of epilepsy and amyotrophic lateral sclerosis, respectively [104,105]. Similarly, a growing body of evidence shows that agonists targeting type II metabotropic glutamate receptors (such as mGluR3) are effective in the treatment of variety of neurological disorders, including pain, addictive disorders, and schizophrenia [99]. In a simple extension, GCPII-specific inhibitors could be equally effective by increasing the extrasynaptic concentration of NAAG and its agonist action at mGluR3 (see below).

4.1.2. Development of GCPII Inhibitors—The first GCPII inhibitors were identified in the early 1990s and included non-hydrolyzable or conformationally restricted NAAG peptide analogs, glutamate derivatives and quisqualic acid [106,107]. Although these inhibitors were weak and lacked specificity, they provided a solid basis for further inhibitor design [29]. Research in the area of inhibitor design expanded during the mid-1990s as more biological information on NAAG became available and the realization that GCPII inhibition had therapeutic value was recognized. A consistent finding throughout the next 15 years of research showed that potent GCPII inhibitors required two functionalities – a glutarate moiety to bind to the C-terminal glutamate recognition site of GCPII and a zinc chelating group to coordinate the divalent zinc atoms at the enzyme's active site. Employing this inhibitor template has led to the identification of several families of potent and selective GCPII inhibitors, exemplified by 2-PMPA, 2-MPPA, and ZJ43 as detailed below.

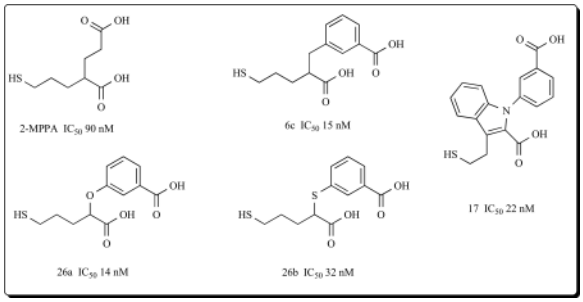
2-PMPA and Phosphonate-Based Inhibitors: The first potent and selective GCPII inhibitor, termed 2-(phosphonomethyl)pentanedioic acid (2-PMPA), was reported in 1996 by Jackson *et al.* [108]. The pentanedioic acid portion of the inhibitor was designed to interact with the glutarate recognition site of GCPII while the phosphonate group was utilized to chelate to the active site zinc ions. 2-PMPA was characterized as a competitive inhibitor with an IC_{50} value of 300 pM with exquisite selectivity having no activity at over 100 different transporter, enzymes and receptors, including several glutamate targets [109]. Subsequently, structure–activity relationship (SAR) studies using 2-PMPA as a template yielded other potent phosphonate-base inhibitors including GPI 5232 [110], VA-033 [111], and phenylalkylphosphonamidates [28].

Phosphinic acid-based GCPII inhibitors have been useful in models of disease to demonstrate the relevance of GCPII as therapeutic target. These inhibitors have shown utility in various preclinical models of disease wherein excess glutamate is presumed pathogenic including ischemia [112–115], pain [116–118], amyotrophic lateral sclerosis [119], seizures [120,121], morphine tolerance [122] and aggression [123]. Unfortunately, poor oral bioavailability and limited brain penetration have precluded this series from advancing to the clinic. Efforts to make 2-PMPA more lipophilic included changes of the γ-acid functionality of glutarate and the preparation of phosphinic acid derivatives. The resulting analogs exhibited lower potency than 2-PMPA even though some inhibited GCPII in the mid nM range. In any case, the lipophilicity of these compounds was still not high enough to improve the pharmacokinetic profile. Current efforts are focused on the synthesis of phosphinic acid prodrugs to enhance oral bioavailability. This strategy has been successfully employed with phosphonate based inhibitors of ACE including Fosinopril [124].

2-MPPA and Thiol-Based Inhibitors: Additional efforts to reduce the polarity of phosphorous-based inhibitors led to thiol-based GCPII inhibitors. Optimization of the number of carbons between the glutarate moiety and the Zn^{2+} binding thiol group resulted in the discovery of 2-MPPA (2-(3-mercaptopropyl) pentanedioic acid) which exhibited nanomolar level potency (IC_{50} = 90 nM).

2-MPPA showed selectivity for GCPII inhibition and most important, it exhibited efficacy in several animal models of disease after oral administration. These included neuropathic pain [125], familial amyotrophic lateral sclerosis [126], painful and sensory diabetic neuropathy [127] and cocaine addiction [128]. 2-MPPA was subsequently used in humans in an exploratory clinical study designed to assess safety and tolerability of GCPII inhibition. 2-MPPA did not provoke any adverse CNS effects and was well-tolerated at plasma concentrations where efficacy was expected to be observed based on animal studies. Even though additional safety and pharmacokinetic studies in healthy subjects were justified, no

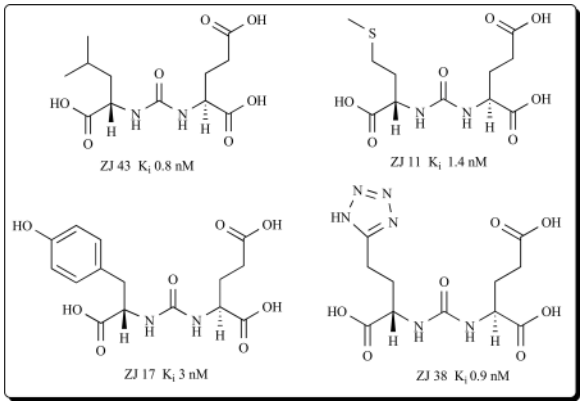
additional studies were carried out because of the relatively low potency of 2-MPPA (estimated human therapeutic dose of ~750 mgs) together with concerns over potential immune reactivity common to thiol containing drugs. Using 2-MPPA as a template, however, more potent thiol based drugs were sought. Thiol inhibitors containing a benzyl moiety at the P1‘ position such as 3-(2-carboxy-5-mercaptopentyl)benzoic acid (6c) were found to be more potent than 2-MPPA (IC_{50} = 15 nM *vs.* 90 nM). Phenoxy and phenylsulfanyl analogues such as 3-(1-carboxy-4-mercaptobutoxy) benzoic acid (26a) and 3-[(1-carboxy-4-mercaptobutyl)thio]benzoic acid (26b) were also found to possess potent inhibitory activities (IC_{50} = 14 and 32 nM, respectively) [23]. Most recently a series of *N*-substituted 3-(2-mercaptoethyl)-1*H*-indole-2-carboxylic acids (e.g 17) with IC_{50} values in the 20–50 nanomolar range were described [129]. Interestingly, these latter compounds represent the first achiral GCPII inhibitors with significant structural differences from NAAG.



ZJ-43 and Urea-Based Inhibitors: Another group of compounds that yielded potent GCPII inhibitors contain urea as the zinc- binding group [26,130–132]. This class of compounds are based on NAAG-like analogs in which the two amino acids are joined through their NH_2 groups by a urea linkage, and utilize both the aspartate binding site (P1 position) in addition to the glutarate moiety (P1’ position) in the enzyme. Noteworthy in this group is ZJ-43 with low nanomolar potency [132] as well as ZJ 11, ZJ 17, and ZJ 38 [26,31,131].

ZJ-43 has shown efficacy in animal models of schizophrenia [132], traumatic brain injury [133], neuropathic pain [134] and inflammatory peripheral pain [135]. Unfortunately, like 2-PMPA and its derivatives, urea-based compounds exhibit very low bioavailability following oral administration and have minimal brain penetration.

Other Classes of Inhibitors: Even though phosphonates, thiols and urea-based compounds have shown limitations as potential clinical candidates, they have invariably demonstrated the relevance of GCPII as a therapeutic molecular target in a variety of diseases that exhibit glutamate excitotoxicity as common denominator. Consequently, robust efforts are being carried out in several laboratories to improve on bioavailability and minimize potential toxicity. These include the development of prodrugs of 2-PMPA derivatives like phosphonate and phosphinate esters [136], more lipophilic and potent thiols [23], and the use of alternative zinc- binding functionalities such as hydroxamates [137]. Additional efforts in our laboratory are focused on creating new GCPII inhibitor structures employing alternative zinc binding groups which when used in other drugs have demonstrated oral bioavailability and drug like character in the clinic. These include 2-aminophenylamides, sulfamides, sulfonamides, imidazoles and other nitrogen containing hetero-cycles.



4.1.3. Utility of GCPII Inhibitors Observed in PNS/CNS Preclinical Models—The utility of GCPII inhibitors has been demonstrated in several animal models of disease whereby enhanced glutamate transmission is presumed pathogenic. These models include inflammatory and neuropathic pain [25,26,116–118,127,134,135,138–145] brain ischemia [25,109,114,146,147] motoneuron disease [148] spinal cord and traumatic brain injury [115,133,149], peripheral neuropathy [127,144,150,151], epilepsy [121] and drug abuse [128,152–157]. These findings are summarized in Table 1.

Importantly the GCPII inhibitor efficacy data have been generated with structurally divergent small molecule compounds including thiol- phosphinic acid-, and urea-based inhibitors as well as NAAG peptide analogs, providing strong evidence for the mechanism of their effect. Studies in GCPII knockout mice also support these inhibitor findings; Bacich *et al* showed that the knockout mice have decreased sensitivity to both central and peripheral nerve damage [158]. From a mechanistic perspective, many GCPII inhibitor effects elicited *in vivo* were blocked by the co-administration of selective group II metabotropic receptor antagonists demonstrating that at least part of the GCPII utility is likely mediated *via* this receptor. Not only has efficacy been observed, but several studies have demonstrated that GCPII inhibition in models of neurological disease is accompanied by a decrease in brain glutamate and a concomitant increase in brain NAAG [109,141]. Importantly in normal animals, however, GCPII inhibition has no effect on basal glutamate transmission [109]. From a therapeutic standpoint, this is ideal. If this holds true in the clinic, GCPII inhibition could limit excess glutamate release and provide neuroprotection without the untoward side effects observed previously with potent glutamate receptor antagonists [159].

4.2. GCPII in Cancer

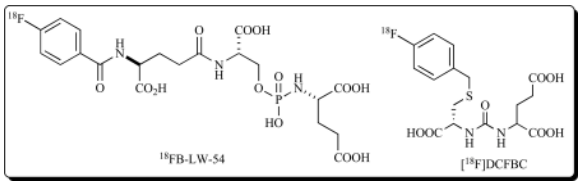
As stated in previous chapters, GCPII is strongly expressed in human prostatic tissue and with gradual increase in staining intensity from the healthy organ through benign prostate hyperplasia, low-grade adenocarcinoma up to metastatic, androgen-independent disease. Given the extremely high levels of expression, a fairly restricted expression pattern and the membrane localization of GCPII, the protein is an ideal candidate for PCa imaging and therapy.

4.2.1. Current Imaging Techniques for PCa—Many recent reviews covering imaging in general and molecular imaging, specifically, in PCa have been published [160–164]. As with most cancers, anatomic imaging with computed tomography (CT) and/or magnetic resonance (MR) imaging remains the mainstay for staging and therapeutic monitoring. The nuclear medicine technique of bone scanning with [^{99m}Tc]methylene diphosphonate (MDP) and single photon emission computed tomography (SPECT), also widely used to detect metastases, is now giving way to [^{18}F]NaF with positron emission tomography (PET), due

to the higher sensitivity of the latter [165]. SPECT and PET are considered molecular imaging techniques in that they detect and enable imaging of the underlying metabolism or a specific protein marker associated with the disease. However, PCa specifically is not particularly amenable to the standard clinical method of PET with [^{18}F]fluorodeoxyglucose (FDG), likely due to the relatively metabolically quiescent nature of PCa vs. other malignancies. Accordingly, a wide variety of new molecular imaging agents and techniques are under development for PCa, such as positron-emitting versions of choline [166,167], 1-amino-3-fluorocyclobutane-1-carboxylic acid (FACBC) [168], 2-deoxy-2-fluoro-β-L-arabinofuranosyl)-5-methyluracil) (FMAU) [168,169], and [^{18}F]fluorodihydrotestosterone (FDHT) [170,171]. Each agent and technique has its niche and reports on a different aspect of the disease. A heavily pursued target for imaging and therapy of PCa – using a variety of affinity reagents – is GCPII, which is also known (in the periphery) as the prostate-specific membrane antigen (PSMA) [32,38,48,49,54,130,172–176].

Since 1996 there has been a murine monoclonal antibody to PSMA radiolabeled with ^{111}In, known as [^{111}In]capromab pendetide or ProstaScint™, used to image PCa [177,178]. However, monoclonal antibodies are somewhat cumbersome as imaging agents due to their long circulation times, which cause significant background radioactivity within the blood pool and other non-target tissues. ProstaScint™ must be administered five days prior to imaging, which is inconvenient, and even then significant blood pool remains obscuring potential malignant lesions within the pelvis [179]. One problem with ProstaScint™ is the fact that it targets an intracellular epitope on PSMA such that the cells must be dead or dying for the antibody to gain access to the target.

Several antibodies to extracellular epitopes of PSMA have since been developed and these are believed at least in part to mitigate the problems associated with the use of ProstaScint™. The most frequently cited of these is the J591 antibody (and its derivatives), which has been used productively to image not only PCa but other solid tumors due to the presence of PSMA within the tumor neovasculature [43,54,180,181]. Reagents based on the J591 antibody (or its humanized derivative huJ591) are clinically the most advanced, and J591 conjugates have been shown to be well-tolerated and non-immunogenic in several clinical trials. Advantageously, in addition to the primary tumor, J591-based reagents are also able to target bone and soft tissue metastases efficiently. J591 radiolabeled with ^{111}In has been used for for SPECT; ^{90}Y, ^{177}Lu or ^{131}I for β-therapy; and ^{213}Bi and ^{225}Ac for α-therapy [180,182–189]. Most recently the J591 antibody has been radiolabeled with ^{89}Zr, taking advantage of the 78.4 h half-life of ^{89}Zr to enable productive tumor imaging [190]. The group lead by Pichler reported preparation of a series of high affinity antibodies against PSMA, their labeling with ^{64}Cu and use for high-resolution PET imaging of prostate cancer xenografts [191–193].



One way to address the problem of poor pharmacokinetics of antibodies for imaging is to use aptamers, which are affinity reagents based on nucleic acids [194]. Aptamers to PSMA have been radiolabeled with the positron emitter ^{64}Cu, but images *in vivo* have not yet been produced [195].

4.2.2. Novel Approaches for GCPII Imaging—Several groups have taken a different approach to circumventing the pharmacokinetic problem by developing low molecular weight agents that bind with high affinity to PSMA, which enable rapid uptake by tumor tissue and washout from non-target sites. The low molecular weight agents fall into two major classes, namely the phosphoramidates and the ureas [31,175,196].

A phosphoramidate peptidomimetic, based on GCPII therapeutics initially developed at ZENECA Pharmaceuticals in the mid 1990's [108], has been radiolabeled with ^{18}F using the N-succinimidyl-4-^{18}F-fluorobenzoate (^{18}F-SFB) prosthetic group, and was able to delineate PSMA+ LNCaP xenografts on small animal PET with 4:1 target (LNCaP tumor) to non-target (PSMA- PC3 tumor) ratios at 1.24% injected dose per gram (ID/g) in LNCaP tumors at 2 h post-injection (compound FB-LW-54) [175]. Recently, Berkman's group reported that depending on the physicochemical characteristics of the P1 moiety, several of their phosphoramidate peptidomimetics feature pseudoirreversible binding to GCPII [27]. Pseudoirreversibility in inhibitor binding has been shown to increase the rate of PSMA internalization and this fact can be exploited for transporting drug into the PSMA-positive cells. No such correlation is available for urea-based inhibitors and it remains to be seen whether this interesting distinction will have any connotations for the future selection of zinc binding groups.

The urea series is originally based on symmetric inhibitors of GCPII discovered by Kozikowski and coworkers [131]. Compounds of the urea class have been radiolabeled with ^{125}I for autoradiography and preclinical imaging, with ^{123}I or ^{99m}Tc for SPECT, and with ^{11}C, ^{68}Ga or ^{18}F for PET [48,49,173,174,197, 198]. Compounds of this class tend to provide images with very high target to non-target ratios and high tumor uptake values, occasionally over 40% ID/g using an isogenic PSMA+/− tumor model system. As with the phosphoramidates, the ureas require a glutamate to bind within the pharmacophore pocket, while an additional carboxylate is required in the S1 site. However, because of a tunnel of 20 Å leading to the surface of the enzyme [7], large functional groups, such as chelating or fluorescent moieties, can be appended to the urea, which is considered the PSMA targeting aspect. The linker from the targeting moiety to the chelator can be used for pharmacokinetic manipulation [173,176]. PSMA targeting compounds labeled with fluorescent species, particularly those that emit in the near-infrared region of the spectrum, can be used for intra-operative surgical guidance [46]. PSMA-targeting ureas labeled with ^{123}I and ^{18}F have recently been used in Phase I clinical trials in patients with PCa to good effect (Fig. 2).

Notably, all successful PSMA-based imaging agents have been used in the periphery for cancer. Because they are highly charged and therefore unable to cross the blood-brain barrier, imaging of GCPII within the central nervous system (CNS) has proved a difficult goal. Nevertheless, efforts to eliminate the tricarboxylic acid motif to enable imaging within the CNS are under way, in analogy with the development of therapeutic agents that penetrate the brain [21]. Motivation for that pursuit derives in part from the ability of N-[N-[(S)-1,3-dicarboxypropyl]carbamoyl]-S-3-[^{125}I] iodo-L-tyrosine ([^{125}I]DCIT) to distinguish patients with schizophrenia from those with unipolar depression in certain brain regions when using *in vitro* autoradiography [199].

4.2.3. GCPII in PCa Therapy—An obvious extension of imaging studies is the use of antibodies and fragments thereof in conjugates for cancer therapy. Several of such reagents have been developed and tested primarily settings. The beta-emitting isotope ^{90}Y has been used to replace ^{111}In in ProstaScint™, and the use of the conjugate resulted in slight improvements in the symptomatology of the disease in PCa patients [200]. More impressive antitumor activity, as determined by the decrease in PSA levels, was obtained by the use

of ^{90}Y-J591 and ^{117}Lu-J591 conjugates, with the ^{117}Lu-J591 offering better and more predictable responses [182,187].

In a complementary approach, several studies used chemical conjugates of antibodies and immunotoxins to target GCPII-expressing cells. Both *in vitro* studies and mouse xenograft models were exploited to test the efficacy of J591 conjugates with ricin A, melitin-like peptide 101, monomethylauristatin E, and maytansinoid 1 [201–204]. Results of these studies showed promise for PCa treatment in preclinical animal models that included significant tumor growth inhibition and even complete ablation of implanted tumors. Future clinical applications might be, however, hampered by inherent problems of such conjugates, including their immunogenicity and the difficulties of producing large and homogenous quantities. These problems might, at least in part, be overcome by harnessing the power of recombinant DNA technology to construct less immunogenic and smaller conjugates. Such a conjugate, comprising a single-chain antibody fragment linked to a truncated form of Pseudomonas exotoxin A, has been engineered and its anti-tumor activity demonstrated in C4-2 tumor xenografts *in vivo* [205,206].

Because GCPII/PSMA is an integral membrane protein with the active site external to the cell, it often serves as a proof-of-principle in developing new nanoparticle-based imaging and therapeutic agents that target tumors with leaky vasculature. One of the first studies to demonstrate nanoparticles targeting an epithelial target used semiconductor quantum dots that employed the J591 antibody to PSMA [207]. Suppression of autofluorescence was a major goal of that study, as the reagents used were for optical imaging. The significance of the study is that particles that could contain a therapeutic payload may be targeted to tumors *in vivo*, despite the target being in epithelium and not within the vasculature, where it is ostensibly much more readily accessible. A variety of combination imaging and therapeutic (theranostic) agents, using several modalities, have been developed recently, including fluorescent, superparamagnetic nanospheres, aptamer-targeted iron oxide nanoparticles loaded with doxorubicin [208,209], and biocompatible nanoparticles carrying preventive as well as therapeutic agents [208,210], some of which are beginning to be functionalized with low molecular weight urea inhibitors for targeting.

A particularly creative application of PSMA targeting is for antibody recruitment. The idea, developed by Spiegel and coworkers, employs a linker between the urea-based targeting moiety and an anti-dinitrophenol (anti-DNP) antibody binding terminus. The so called antibody recruiting molecule to prostate cancer (ARM-Ps) link the anti-DNP antibody to the prostate tumor cell, where it can be cleared by the immune system by virtue of the immune-stimulating capacity of DNP [211]. Interestingly, in developing the ARM-P technology, a new arene-binding site (for the DNP moiety) was uncovered in PSMA, enabling the synthesis of further high-affinity ligands [30]. This technology provides an excellent example of how deeper mechanistic understanding of the biology of even a well studied system such as GCPII/PSMA in the periphery can inform further chemical synthesis of new drugs and other reagents.

5. FUTURE PERSPECTIVES

Human GCPII has shown its utility in a variety of preclinical and clinical settings ranging from the treatment of neurological disorders to imaging and therapy of prostate cancer and solid malignancies. There are, however, many challenges still lying ahead. In the area of neurological diseases, it is imperative to identify and develop orally available GCPII inhibitors that could efficiently penetrate the blood-brain barrier and target GCPII residing in the CNS. Additionally, isoform-specific small molecule ligands that are able to discriminate between GCPII and its paralogs (e.g. GCPIII) might be needed for truly

specific imaging of GCPII in human brain. As for cancer-related applications with the focus on therapy, it would be highly desirable to unravel physiological function(s) of GCPII in cancerous tissues including tumor neovasculature so that a more rational approach could be applied in this area. Additionally, more comprehensive biological and structural information is needed on GCPII paralogs and mammalian orthologs (including interspecies differences) that could again simplify and rationalize the development of modalities targeting GCPII under various (patho)physiological conditions. The development of future GCPII-specific modalities (small-molecule probes as well as macromolecules targeting GCPII) will be instrumental to decipher the role of GCPII in both healthy and diseased tissues. We believe that the unique characteristics of GCPII render this enzyme an attractive target for both basic and applied research.

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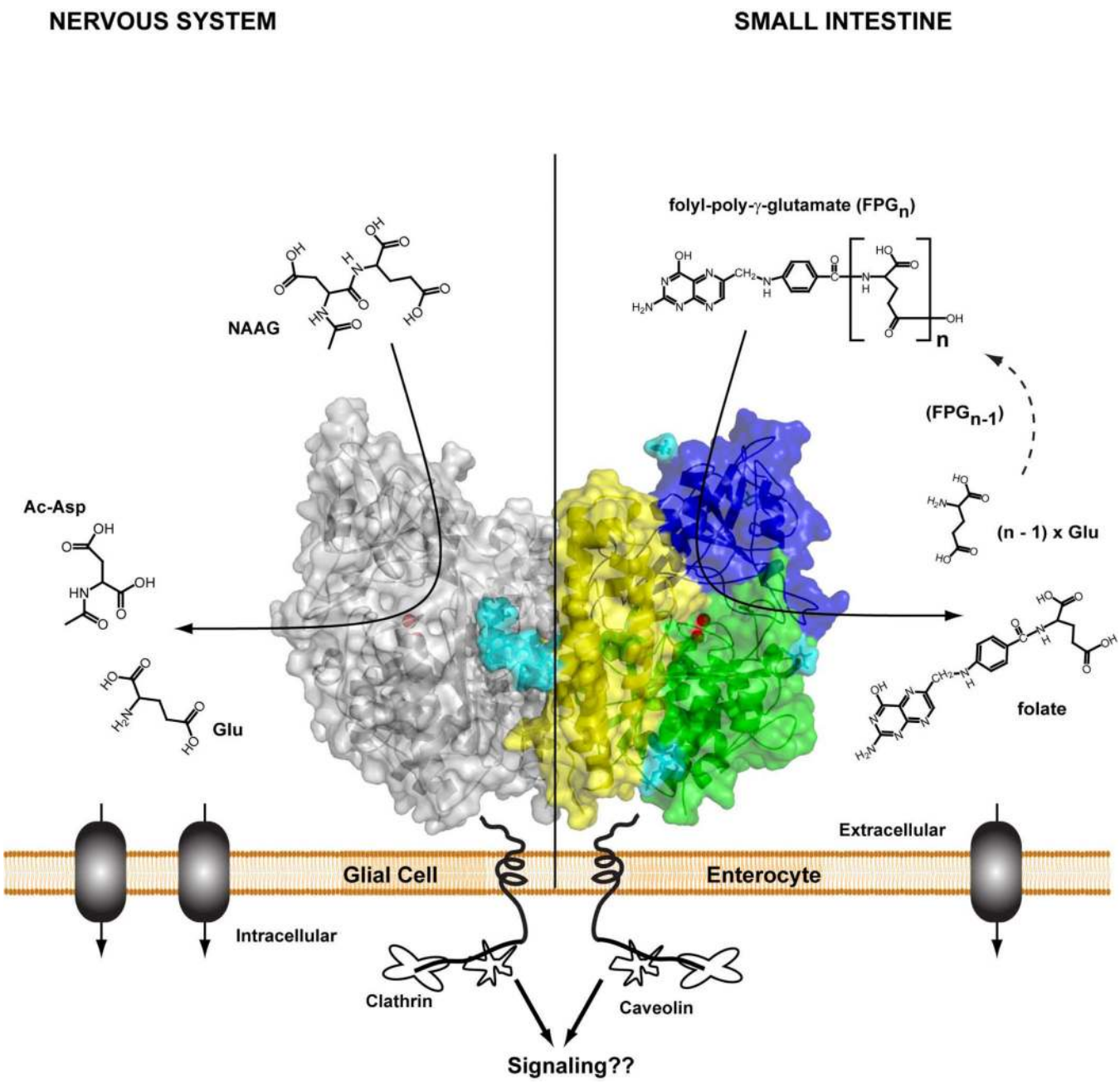

Fig. (1).

Homodimer of human GCPII (crystal structure) tethered to the biological membrane. One monomer shown in semitransparent surface representation with individual domains of the extracellular part colored green (protease domain; amino acids 57 – 116 and 352 – 590), blue (apical domain; amino acids 117 – 351), and yellow (C-terminal; amino acids 591 – 750); the second monomer is colored gray. N-linked sugar moieties are colored cyan, and the active-site Zn^{2+} ions are shown as red spheres. **Left panel** – residing at the plasma membrane of astrocytes /schwann cells, GCPII catabolizes NAAG, the most prevalent peptidic neurotransmitter in the mammalian nervous system. N-acetylaspartate and glutamate, the reaction products, are selectively transported into glial cells, metabolized and reused for NAAG synthesis in neurons. **Right panel**– GCPII (or folate hydrolase) at the

plasma membrane of enterocytes in the proximal jejunum sequentially hydrolyzes the C-terminal γ-glutamate tail of dietary folates, finally leaving folate-monoglutamate, which can be then transported transcellularly into the blood stream.

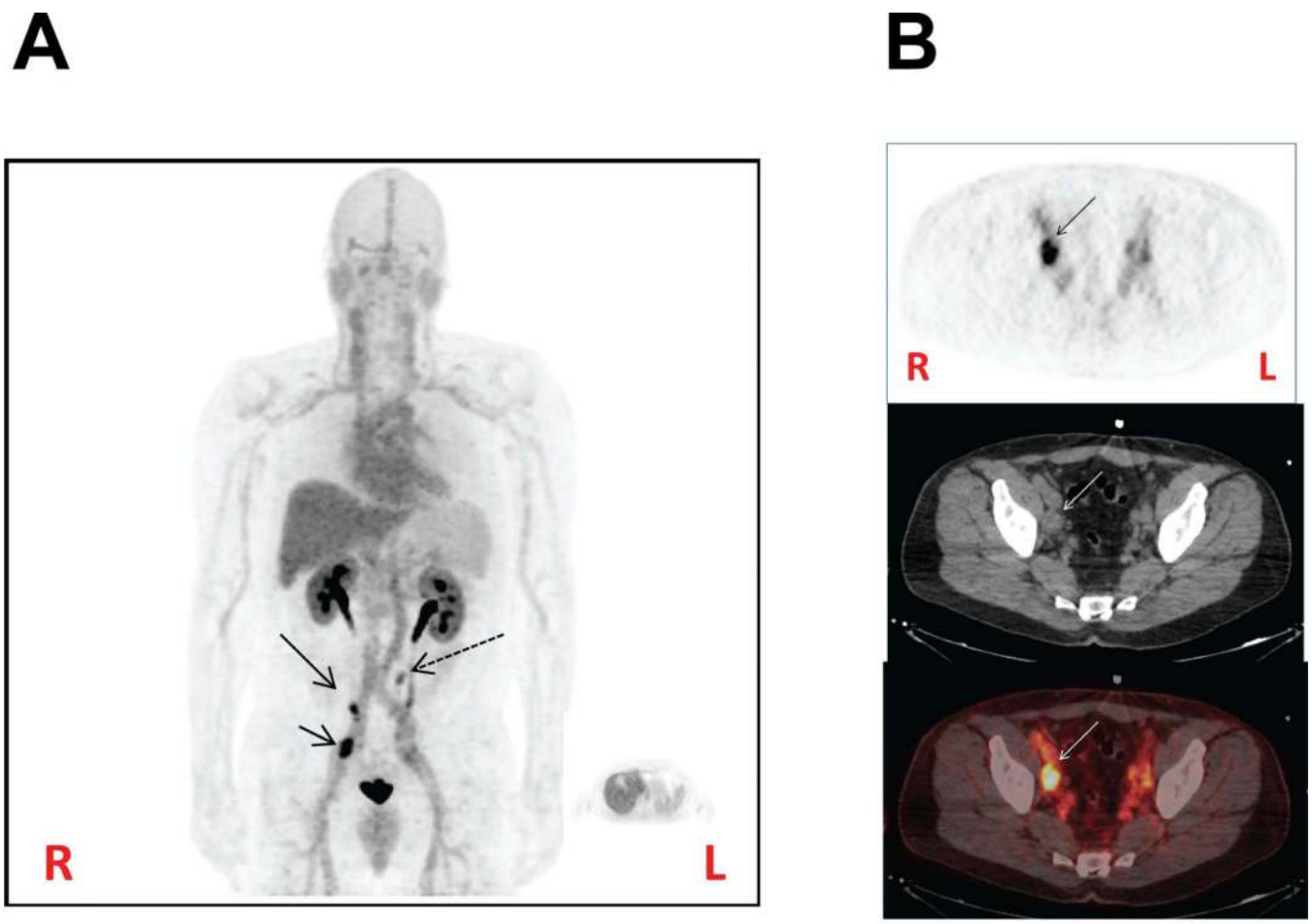

Fig. (2).

Human PET imaging with [^{18}F]DCFBC. (**A**) **Coronal view**. Note increased radiopharmaceutical uptake in an enlarged right external iliac lymph node (short arrow), a small right external iliac node (long arrow) and a small left periaortic node (dashed arrow). [^{18}F]DCFBC, like all of the hydrophilic GCPII inhibitors of the urea class, is excreted almost exclusively through the urine. The large pelvic focus of uptake in the midline is due to radiotracer in the bladder. (**B**) **Axial view**. Clockwise from upper left: PET, CT and fused PET/CT images depicting the right external iliac lymph node (arrow). These foci of increased radiopharmaceutical uptake are most likely due to metastases from prostate cancer. R = right, L = left.

Table 1

INHIBITOR USED	MAJOR FINDING	REF
PAIN		
2-PMPA, i.t.	Antinociceptive effect in rat formalin but not hot plate model	[118]
2-PMPA, i.t.	Reduction of mechanical allodynia induced by paw carrageenan injection; no effect on post-op pain or thermal injury	[117]
Cmpd 34	Reduction of thermal hyperalgesia in CCI model of neuropathic pain	[25]
2-PMPA, i.p.	Reduction of allodynia and ectopic discharge activity of afferent nerves in partial nerve ligation model	[116]
GPI 5232 i.p.	Reversal of thermal hyperalgesia in BB/W diabetic rats following 6 mo dosing	[144]
2-MPPA, p.o.	Reduction of thermal hyperalgesia in CCI model of neuropathic pain	[140]
2-PMPA, applied directly	Inhibition of input, post-discharge, C- and Aδ-fibre-evoked responses after carrageenan inflammation	[139]
Cmpd 8d (urea) i.t.	Reduction of the response to inflammatory pain evoked by footpad injection of formalin	[26]
ZJ-11, 17 and 43, i.t. and i.v.	Reduction of mechanical allodynia in formalin and the partial sciatic nerve ligation models blocked by co-administration of a group II mGluR antagonist	[134]
ZJ-43, i.p.	Reduction of mechanical allodynia in a bone cancer model; effect was antagonized by group II mGluR antagonist	[145]
2-MPPA, p.o.	Reversal of thermal hyperalgesia in BB/W diabetic rats following 6 mo dosing	[127]
2-PMPA, i.p.	Reduction of allodynia in CCI model with brain levels of 2PMPA (30uM) and NAAG (3uM)	[141]
ZJ43 and 2-PMPA, footpad	Reduction of pain responses in carrageenan and formalin models blocked by co-administration of a group II mGluR antagonist	[135]
ZJ43 & 2-PMPA i.c.v.	Dose dependent analgesia blocked by co-administration of a group II mGluR antagonist in mouse formalin model	[143]
ZJ-43, i.p.	Prevention of the development of mechanical allodynia and reduction of the enhanced current output in the central nucleus of amygdala in mouse formalin model	[138]
STROKE / ISCHEMIA		
2-PMPA, i.p.	Neuroprotection following middle cerebral artery occlusion; concomitant increase in brain NAAG and attenuation of ischemia-induced rise in glutamate	[109]
2-PMPA	Neuroprotection in rat cerebellar neurons against hypoxia and NMDA	[146]
2-PMPA, i.p.	Neuroprotection of retinal ganglion cell survival in an ischemia-reperfusion model of C57BL/6 mouse eyes	[115]
GPI 5232, i.v.	Reduction of brain infarction and amelioration of pathological EEG changes following middle cerebral artery occlusion	[147]
GPI 5232, i.v.	Neuroprotection in a middle cerebral artery occlusion model of cerebral ischemia	[25]
ALS / MOTONEURON DISEASE		
2-MPPA, p.o.	Protection against motoneuron death in G93A familial ALS transgenic mice	[148]
PERIPHERAL NEUROPATHY		
GPI 5232, i.p.	Partial prevention of hyperalgesia, nerve conduction slowing, axonal and morphological abnormalities in BB/W rat model of Type I diabetes	[144]
2-PMPA	Protection against glucose-induced neuronal death and neurite degeneration in dorsal root ganglion neurons; blocked by group II mGluR antagonist	[150]
2-MPPA, p.o.	Improvement of established hyperalgesia, nerve conduction velocity abnormalities and myelinated fiber atrophy in BB/W rat model of Type I diabetes	[127]
2-MPPA, p.o.	Protection of the nerve conduction velocity and morphological abnormalities induced by several chemotherapies including cisplatin, paclitaxel, bortezomib	[151]
EPILEPSY / SEIZURES		

INHIBITOR USED	MAJOR FINDING	REF
2-PMPA, i.p.	Protection against the development of cocaine-kindled seizures; no effect on acute challenges with pentylenetetrazole, bicuculline, N-methyl-D-aspartate, maximal electroshock	[121]
DRUG ABUSE		
2-PMPA, i.p.	Reduction of ethanol consumption in alcohol-preferring (P) rats	[152]
2-PMPA, i.p.	Attenuation of the development of sensitization to the locomotor-activating effects of cocaine; no antagonism of the acute effects of cocaine	[155]
2-PMPA, i.p. and 2-MPPA, p.o.	Attenuation of the acquisition and expression of the conditioned place preference response to cocaine but not food	[128]
2-MPPA, p.o.	Prevention of the development of morphine tolerance without affecting acute morphine antinociception; blocked by co-administration of a group II mGluR antagonist	[153]
2-PMPA, i.p.	Inhibition of cocaine's rewarding effects as assessed by i.v. cocaine self-administration and intracranial electrical brain-stimulation reward; blocked by co-administration of a group II mGluR antagonist	[156]
2-PMPA, i.p.	Attenuation of cocaine-induced reinstatement of drug-seeking behavior *via* blocking increases in dopamine and glutamate in the nucleus accumbens; blocked by co-administration of a group II mGluR antagonist	[157]
2-MPPA, p.o.	Attenuation of cocaine-induced reinstatement of drug-seeking behavior in rats; no effect on sucrose-seeking behavior; blocked by co-administration of a group II mGluR antagonist	[154]
BRAIN AND SPINAL CORD INJURY		
ZJ-43, i.p.	Protection of degenerating neurons and astrocyte loss following lateral fluid percussion model of traumatic brain injury in rats; effect abolished by co-administration of a group II mGluR antagonist	[133]
ZJ-43, i.p.	Increase in dialysate NAAG and reduction in glutamate, aspartate and GABA levels in hippocampus following fluid percussion injury	[149]
2-PMPA	Protection against dynorphin A-induced ischemic spinal cord injury in rats assessed by reduction of cerebrospinal fluid glutamate and improvement in motor scores	[115]