Glutamate carboxypeptidase II

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GLUTAMATE CARBOXYPEPTIDASE II

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Abstract:

Glutamate carboxypeptidase II (GCP II) is a homodimeric dinuclear zinc carboxypeptidase catalysing the hydrolysis of substrates bearing L-glutamate at the C-terminal position. The GCP II-catalysed hydrolysis of the neuropeptide *N*-acetyl-L-aspartyl-L-glutamate is considered an important source of glutamate, whose excesses are associated with various neurological disorders. Additionally, an increased expression of GCP II is often detected in cancer. In this context, GCP II is currently a pharmacological and diagnostic target being pursued in the clinic for the regulation of glutamate and for imaging purposes. An overview on GCP II, including its reaction mechanism, its biological localisation and its physiological roles, is provided herein. The main classes of GCP II inhibitors, as well as recent advances in the development of potential therapeutic and diagnostic tools based on the inhibition and/or detection of GCP II are also described in this chapter.

Key Words: *N*-Acetyl-L-aspartyl-L-glutamate (NAAG); Reaction mechanism; Inhibitors; Small molecules; Antibodies; Cancer; Prostate; Nervous system, Inflammation; Brain; Imaging; Theranostic.

INTRODUCTION

The type-II membrane glycoprotein glutamate carboxypeptidase II (EC 3.4.17.21), known as GCP II, is a homodimeric dinuclear zinc carboxypeptidase with the highest expression levels found in the nervous and prostatic tissue [1,2].

GCP II catalyses the hydrolysis of endogenous substrates bearing L-glutamate at the C-terminal P1' position. One of these substrates is the abundant brain peptide *N*-acetyl-L-aspartyl-L-glutamate (α-NAAG), whose cleavage provides *N*-acetyl aspartate and glutamate. GCP II also catalyses the hydrolysis of C-terminal glutamate residues of folylpoly-γ-L-glutamate (also known as folate polyglutamate and as pteroylpoly-γ-L-glutamate). Owing to these catalytic activities, GCP II has also been formerly referred as *N*-acetylated-α-linked acidic dipeptidase (NAALADase) and as folate hydrolase (FolH1).

Glutamate carboxypeptidase II is also known as prostate-specific membrane antigen (PSMA). It is worth remembering that this membrane-bound exopeptidase (EC 3.4.17.21) is different from the prostate specific antigen (PSA, EC 3.4.21.77), which refers to kallikrein-related peptidase 3 [1,3].

GCP II BIOLOGICAL LOCALISATION

Both *in vitro* immunoactivity and enzymatic essays showed that significant differences exist in the localisation of GCP II between different species [3-11]. According the above-mentioned studies, in healthy humans GCP II is mainly expressed in prostate (secretory acinar epithelium), central and peripheral nervous system (astrocytes and Schwann cells respectively), kidney (proximal tubules) and small intestine (brush border membranes of jejunum) [3-9,12-18]. A lower expression was also detected in testis, ovaries, bladder, pancreas, heart, salivary glands, skin, liver, lung, colon [4-9,16,19]. *In vivo* researches, carried out by using GCP II small ligands or labelled antibodies, showed a restricted expression of the enzyme. This might be due to a limited accessibility of GCP II for test ligands *in vivo* or to the masking of the sites of interaction by other molecules or post-

translational modification [8,20-24]. The *N*-acetyl-aspartyl-glutamate (NAAG) distribution seems to influence the CGP II distribution in most regions of the human body [3].

Nervous system

NAAG is the majorly distributed peptide transmitter in the mammalian brain and it is also the one with the main concentration (mM). It is synthetized in neurons and stored in presynaptic axon terminals in vesicles. [25]. In the nervous tissue NAAG exhibits a negative modulatory effect while glutamate has an excitatory role [3,26].

NAAG is released in the synaptic cleft after calcium-dependent depolarization, then it quickly diffuses in the extra synaptic space where it can take two different ways. In the first case NAAG can act as an agonist at the metabotropic glutamate receptor 3 (mGluR3) on the presynaptic nerve terminal and astrocytes. By activating mGluR3, NAAG leads to a decrease in the cellular concentration of the second messengers cAMP and cGMP (*via* a G-protein coupled pathway). In the presynaptic nerve this causes a reduction in the amount of glutamate that will be released upon further nerve stimulation. In the astrocytes the link of NAAG to mGluR3 supports the production and secretion of neuroprotective factors such as the transforming growth-factor β [27-31]. Alternatively, NAAG can undergo hydrolysis by GCP II into *N*-Ac-Asp and glutamate, that are transported into astrocytes and oligodendrocytes. In this case, NAAG is no longer able to perform the agonist activity on mGluR3 [8].

The GCP II enzymatic activity – and its possible regulation – may determine if the excitatory or inhibitory effects would prevail after the NAAG release in neural system [3].

“Glutamate excitotoxicity” is a phenomenon due to the non-physiological increase in the glutamate concentration in synapsis. This causes hyperactivation of ionotropic glutamate channels NMDAR with consequent excessive calcium influx and triggering of several cellular responses including nitric oxide overproduction, ionic homeostasis imbalance, caspase activation, mitochondrial dysfunction and associated free radical generation, which result in the necrotic or apoptotic death of

the neuron. Consequently, neuronal dysfunction and degeneration both acute and chronic, including stroke – brain ischemia [8,32], amyotrophic lateral sclerosis, Alzheimer's and Parkinson's diseases, traumatic brain injury [8,33,34], inflammatory and neuropathic pain [35-37], peripheral neuropathy [38,39], epilepsy [40] might occur.

A variety of studies on animal models showed that a lower stimulation of ionotropic glutamate channels might ameliorate serious pathological disorders both of peripherical and central nervous system [8]. This is also supported by the evidence of GCP II dysregulation in several neurologic and psychiatric disorders that involve glutaminergic neurotransmission [3,41-43].

The inhibition of GCP II increases the intact NAAG part and decreases the glutamate concentration. Furthermore, under GCP II inhibition the mGluR3 activation leads to additional reduction of released glutamate and to the secretion of protective peptides. Supporting this findings, GCP II knock-out mice showed reduced sensitivity to central and peripheric nerve damage [44] and proved to be less sensitive to traumatic brain injury in terms of neuronal loss, oxidative stress and apoptosis [45,46] The involvement of the mGluR3 receptors was confirmed in animal models of memory, Alzheimer's, and ethanol intoxication; GCP II inhibition was found to improve cognition and to reduce the cognitive and motor deficits induced by ethanol [47].

The attempt to employ NMDAR (*N*-methyl-D-aspartate receptor) antagonist for therapeutical purposes has failed. In this context, the employ of small molecules as GCP II specific inhibitors might be effective, *via* the increase of extrasynaptic NAAG concentration and its agonist action on mGluR3. In several preliminary *in vitro* and animal model studies, the utility of GCP II inhibitors has been demonstrated with multiple potential clinical applications as discussed by different authors [8,48,49] for inflammatory and neuropathic pain [8,50-52], peripheral neuropathy [8], motoneuron disease [8], cognitive impairment in multiple sclerosis [53], brain ischemia [8], spinal cord and traumatic brain injury [8], perinatal injury [54], epilepsy [8], schizophrenia [55], ethanol intoxication [47] and drug abuse (reducing self-administration and priming induced drug seeking)

[8,56-59]. The efficacy of GCP II inhibition has also been confirmed in preclinical models of neurological disorders, where glutamatergic excitotoxicity plays a role [60,61].

Very recently, the importance of GCP II inhibition as a potential strategy for the treatment of cognitive disorders associated with aging and/or neuroinflammation has been endorsed in studies with animals [62].

Kidney

In kidney GCP II is located in the brush border of the proximal convoluted tubules where it has a role (still not fully understood) in the selective circulating peptides reabsorption [3,5].

Small Intestine

In the brush border of small intestine cells GCP II cleavages the dietary poly-γ-glutamyl folates to their mono-glutamyl form by removing the γ-linked glutamate. Mono-glutamyl folates can then be actively absorbed by specific carriers [3,8]. This activity is critical in human nutrition since humans require folates and folate metabolism has been reported to be possibly influenced by GCP II polymorphism. However, further epidemiologic studies are needed to clarify the actual role of GCP II in folate metabolism [8].

GCP II Structure and Reaction Mechanism

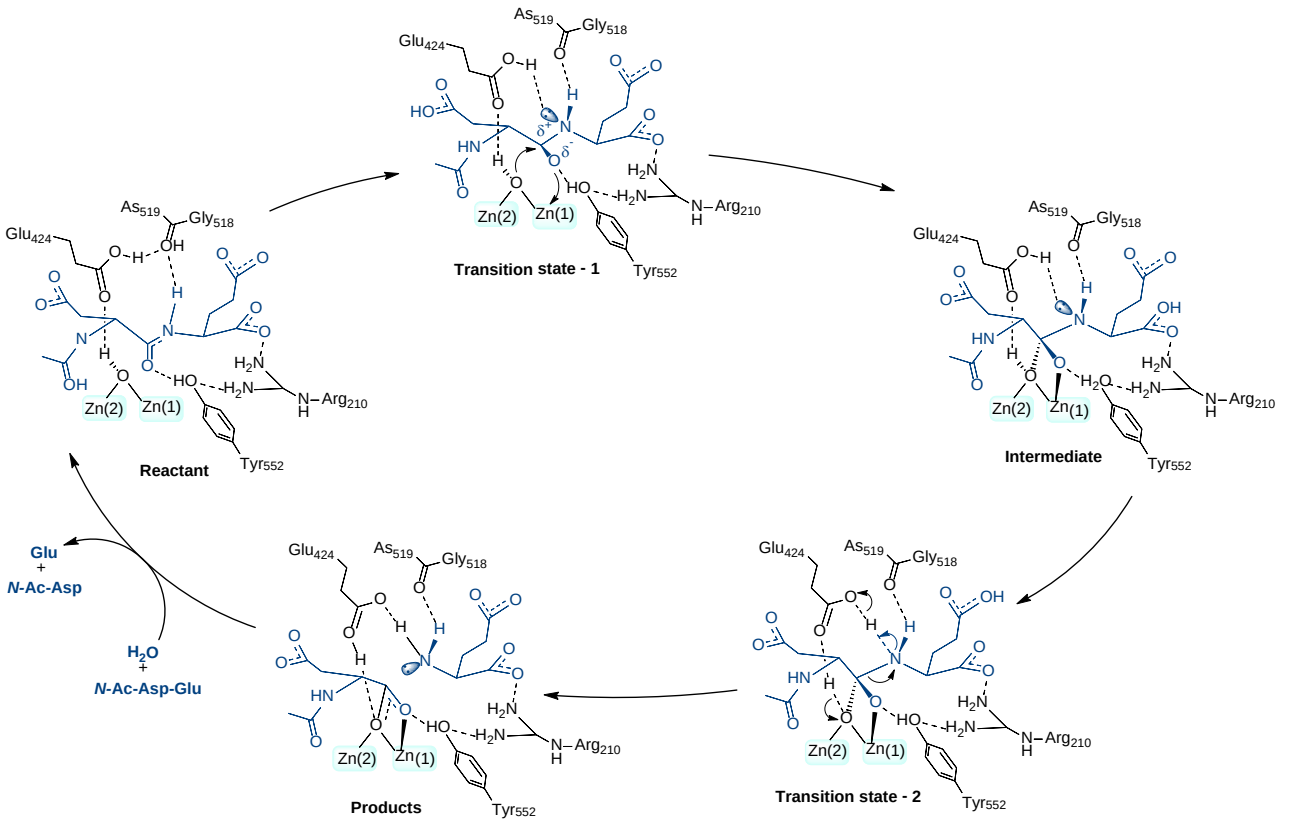
GCP II is characterised by the presence of two Zn^{2+} ions in its active site within a (μ-aquo)(μ-carboxylato)dizinc(II) core. The two zinc ions are coordinated by two histidines (His377 and His 553), two aspartates (Asp387, bridging the two Zn^{2+} ions, and Asp453, which binds one Zn^{2+} ion in a bidentate mode), and one glutamate residue (Glu425). The zinc-zinc distance is of 3.3 A. In its substrate-free form, the two zinc ions of GCP II are bridged asymmetrically by a bidentate water or hydroxyl ligand. Thus, each of the two Zn^{2+} ions have the classical tetrahedral coordination sphere.

Although the mechanism of GCP II is not understood in details, some information can be extrapolated from studies performed on other structurally related metallopeptidases. For example,

experimental and computational data are available on aminopeptidase from *Aeromonas proteolytica* (AAP) and *Streptomyces griseus* (SGAP) [63,64].

Such studies suggested that in GCP II the bridging oxygen is donated by a hydroxide anion and that the Glu424 (equivalent of Glu 151 in AAP) is a proton shuttle between the bridging water molecule (deprotonated upon coordination to Zn^{2+}) and the scissile peptide bond. Additionally, docking studies performed on model dipeptides indicated that the substrate is bound to the dizinc cluster through the terminal amino functionality rather than the carbonyl moiety of the peptide bond. The bridging hydroxide can perform the nucleophilic attack at the carbonyl group without being shifted to the terminal position, which imply coordination at the Zn(1) only. DFT studies on the model of the active site of AAP also highlighted that the proton transfer from Glu151 to the nitrogen atom of the peptide bond is reasonably the rate-determining step. The following steps of the cycle, including the proton transfer from OH^- to Glu151 and the cleavage of the peptide bond, involve a sequence of intermediates and transition states with small reaction barriers. The catalytic Zn(1) ion stabilises the anionic tetrahedral intermediate, whereas the cocatalytic Zn(2) is involved in the binding of the substrate and in the orientation of the peptide bond towards the nucleophile [65]. Thus, the main role of the Zn(1) ion is the binding and the activation of a water molecule for the nucleophilic attack onto the scissile peptide bond. On the other hand, the Zn(2) ion mainly polarises the carbonyl functionality of the peptide bond, compensating for the absence of an oxyanion hole in GCP II. Both Zn ions act stabilising the *gem*-diol transition state [66]. The distance between the two Zn^{2+} ions increases from 3.3 A to 3.6-3.8 A in complexes with transition-state-analogue inhibitors [66,67]. A similar scenario may also occur in the *gem*-diol transition state of bound substrates.

Based on crystallographic, biochemical, and computational evidences, Lubkowski *et al*. proposed a detailed mechanism of substrate hydrolysis by human GCP II (Figure 1) [68].



Insert Figure 1 here

Caption: Figure 1. Reaction mechanism of GCP II by Lubkowski *et al*. [68]

Because two of its lone pairs are occupied in the coordination with the two zinc ions of the active pocket of the enzyme, the hydroxide anion of GCP II is significantly less nucleophilic than a free hydroxide anion. The direct attack of the OH^- at the carbonyl carbon of the peptide bond is therefore disfavoured and the reaction reasonably proceeds concertedly with the proton activating the nitrogen atom of the peptide bond and the concomitant formation of a metastable tetrahedral intermediate. In this regard – as mentioned above – Glu424 behaves as a general base/acid which shuttles the first proton from the activated water to the nitrogen atom of the peptide bond and then accepts the second proton from the bridging OH^-.

Several interactions take place between *N*-Ac-Asp-Glu and the substrate binding cavity of GCP II. The β-carboxylate group of the Asp residue of the substrate is involved in ionic interactions with the guanidinium groups of Arg534 and Arg536. The presence of hydrogen bonding interactions

with Asn519 and with two water molecules is also suggested. The carbonyl oxygen of the acetyl group is engaged in interactions with the side chain of Arg536, Asp453, Asn519; additionally, the methyl group protrudes into a pocket delimitated by the side chains of Ile386, Asp387, Ser454, Glu457, and Tyr549 [68].

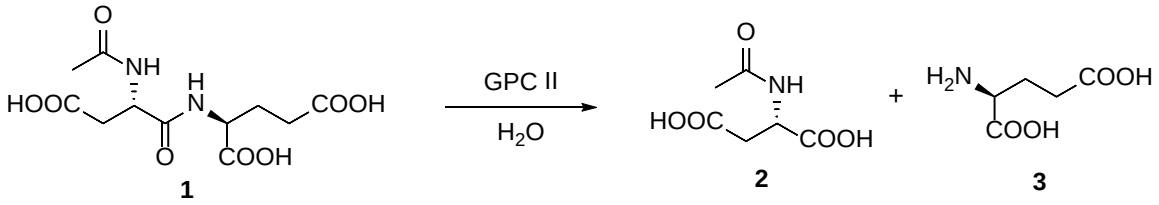
According DFT calculation-based studies, in transition state-1 (Figure 1), the hydrogen of the hydroxyl group of Glu24 is in the near-attack conformation and the Zn─Zn distance is elongated (from 3.3 A to 3.5 A). The carbonyl oxygen of the peptide bond is stabilised by Zn(1). Following the transition state-1, the system evolves towards the formation of the intermediate, which is structurally characterised by i) the elongated Zn─Zn distance, ii) a newly formed C─O bond between the oxygen atom of the bridging hydroxide and the carbon atom of the peptide bond of the substrate, iii) the stabilisation of the carbonyl oxygen of the substrate by Zn(1) and Tyr552, iv) the repositioning of the protonated hydroxyl group of Glu424 (Figure 1).

The transition state-2 (reasonably the rate-determining one) foresees the elongation of the hydrolysed C─N bond. The reaction proceeds through a proton transfer from the hydroxyl group of Glu424 to the nitrogen atom of the peptide bond, a proton transfer from OH^- to Glu424 and, finally, the cleavage of the peptide bond. Following the transition state-2, the addition of a water molecule – which replaces the bridging hydroxide used in the hydrolysis – leads to the dissociation of *N*-Ac-Asp from the active site (Figure 1). According to DFT calculations the release of *N*-Ac-Asp from the active site is promoted by the “pull effect” of several arginine residues (Arg463, Arg511, Arg534, Arg536) located in the exit channel of GCP II [66]. At this stage, the glutamate residue – which is the second product of the reaction – likely remains bound in the active pocket of the enzyme [68].

GCP II INHIBITION

Inhibitors of GCP II have been widely studied. Their physiological role and the possibility to use GCP II inhibitors for therapeutic purposes (*i.e.*, neurological disorders, cancer) have been extensively reviewed [3,8,61,69-75].

Excess of glutamate is associated with various neurological disorders, as stroke, amyotrophic lateral sclerosis, spinal cord injury, epilepsy, chronic pain. The formation of glutamate by GPC II-catalysed hydrolysis of *N*-acetylaspartylglutamate **1** (NAAG) is considered an important source of glutamate **3** (Figure 2). Thus, besides conventional therapeutic approaches for the treatment of the above-mentioned diseases (use of small molecules to block postsynaptic glutamate receptors or upstream reduction of presynaptic glutamate), the use of potential GPC II inhibitors as therapeutic agents, to prevent glutamate release, was deeply investigated.



Insert Figure 2 here

Caption: Figure 2. GPC II-catalysed hydrolysis of *N*-acetylaspartylglutamate (NAAG, **1**).

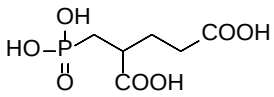
The first chemicals capable to inhibit GCP II were adaptable oxoanions like phosphate and sulfate, as well as bidentate metal ion chelating agents like EDTA and EGTA (CLAN MH 1).

Initially the attention was focused towards the synthesis of peptide analogues of NAAG **1** to evaluate their capability to inhibit the hydrolysis by NAALA dipeptidase (*N*-acetylated alpha-linked acidic dipeptidase). It was found that *N*-fumaryl-L-glutamic acid was the most active GCP II inhibitor (IC_{50} value of 0.8 μM) [76]. The kinetics of NAAG hydrolysis by the cloned human enzyme were also evaluated, showing a pharmacologic profile very similar to the endogenously expressed GCP II activity [77]. Glutamate and quisqualic acid derivatives were identified as GCP II inhibitors, active at μM concentration [22,23,78]. While peptide-based inhibitors were not used in evaluating the therapeutic usefulness of GPC II inhibition, their investigation was important to gain

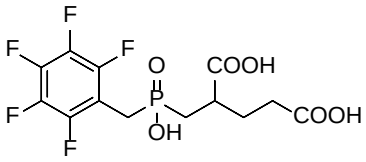
insight into the structural requirements for inhibition of this enzyme. It was showed that the spacing linker groups in the C-terminal glutamate residue are significant for binding to the active site of the enzyme. Later on, effective GCP II inhibitors mainly belonging to the classes of i) phosphonate-based inhibitors (IC_{50} 300 pM), ii) thiol-based inhibitors (IC_{50} 90 nM), iii) hydroxamate-based inhibitors, iv) urea-based inhibitors (IC_{50} 20 nM), and v) sulfamide-based inhibitors have been described [8,70,73,79,80].

2-(Phosphonomethyl)pentanedioic acid (2-PMPA) inhibitors

Relevant results were reported by the Jackson's group, who first identified phosphorus-based inhibitors, such as **2-PMPA** (2-(phosphonomethyl)pentanedioic acid) (Figure 3). **2-PMPA** proved to be a very active compound with a K_i = 0.275 nM [81], with high selectivity for GCP II and high aqueous solubility/stability [32]. **2-PMPA** is a competitor inhibitor; its pentanedioic acid portion interacts with the glutarate recognition site of GCP II and the phosphonate group chelates the zinc ions at the active site.



2-PMPA



GPI 5232

Insert Figure 3 here

Caption: Figure 3. **2-PMPA** GCP II inhibitors.

Slusher and coworkers reported the therapeutic utility of GCP II inhibition by **2-PMPA**, able to protect against ischemic injury and to reduce the ischemia-induced rise in glutamate, thus contributing to neuroprotection in animal models [82-85]. However, the polar nature of **2-PMPA** caused a poor pharmacokinetic profile, thus limiting its use as a therapeutic drug. The fluorinated aryl-derivative **GPI 5232** (Figure 3) also behaved as an effective inhibitor *in vitro* and *in vivo*,

reducing brain injury in animal models [86]. Interestingly, the (*S*)-enantiomer of **GPI 5232** was more potent with respect to the (*R*)-enantiomer [87]. Crystal structure of the extracellular part of GCP II in complex with **2-PMPA** was described [67].

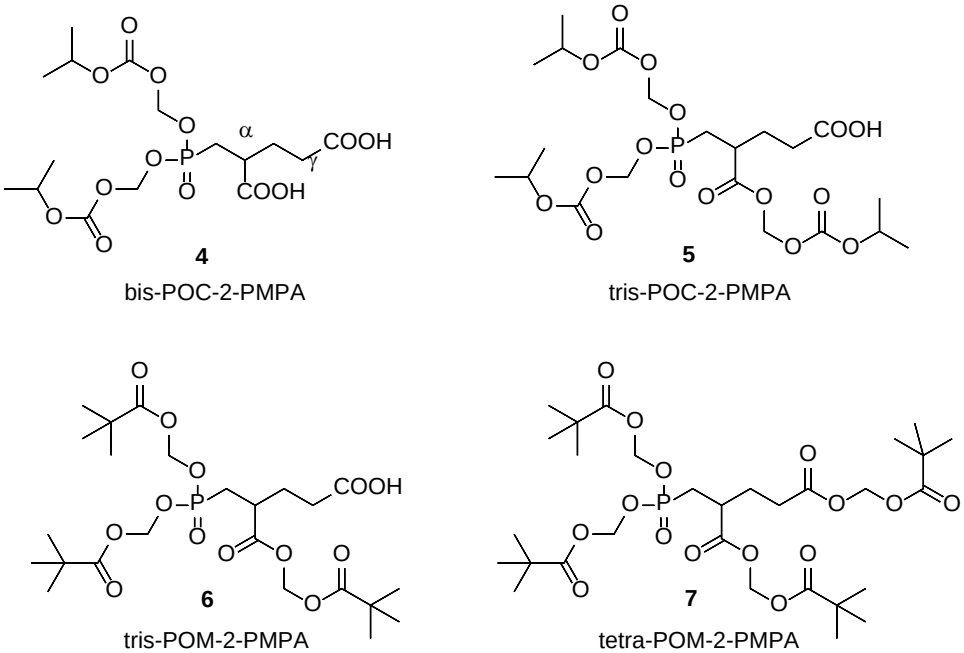
The activity of **2-PMPA** in several preclinical models of neurological disorders associated with glutamatergic excitotoxicity was recently reviewed [61]. Additionally, **2-PMPA** showed a broad and potent analgesic and neuroprotective effects in a variety of preclinical studies [8,52,88]. A novel gut-restricted GCP II inhibitor IBD3540 with high oral anti-colitis efficacy in mouse models with a promising preclinical safety profile was also reported [89].

Besides the **2-PMPA** usefulness in several preclinical models, its clinical use was rather hampered for its high polarity and therefore for its low oral bioavailability. Indeed, **2-PMPA** and related structures were effective in treatment and useful to prove the role of GCP II in models of disease where an excess of glutamate is thought to be pathogenic, including amyotrophic lateral sclerosis, pain, ischemia, seizures, morphine tolerance, and aggression [8]. However, the low oral bioavailability and the limited brain penetration significantly limited the advance of the above-mentioned compounds in clinical tests. Very recently, the therapeutic potential of **2-PMPA** in the treatment of Alzheimer's disease in animal models [47], glioblastoma - *in vitro* [90] and Amyotrophic Lateral Sclerosis - *in vitro* [91]was confirmed. New opportunities of **2-PMPA** as a possible nephron-protective strategy in PSMA-targeted prostate cancer radiotherapy also arose [92,93], giving new input to the research on these molecules.

Several attempts were performed in order to increase the oral bioavailability and the lipophilicity, thus improving the possibility for these molecules to cross the blood brain barrier) [8]. At first the new synthetized molecules showed a lower potency (in the nM range) without any significant improvement of the pharmacokinetic. The intranasal administration seemed to offer in some case a valid alternative to deliver therapeutic concentrations in the brain [94,95]. The synthesis of prodrugs as an alternative route has also been followed [61], with encouraging results in animal models for

some **2-PMPA** prodrugs in terms of stability, pharmacokinetics and delivery to plasma and brain [61,95,96].

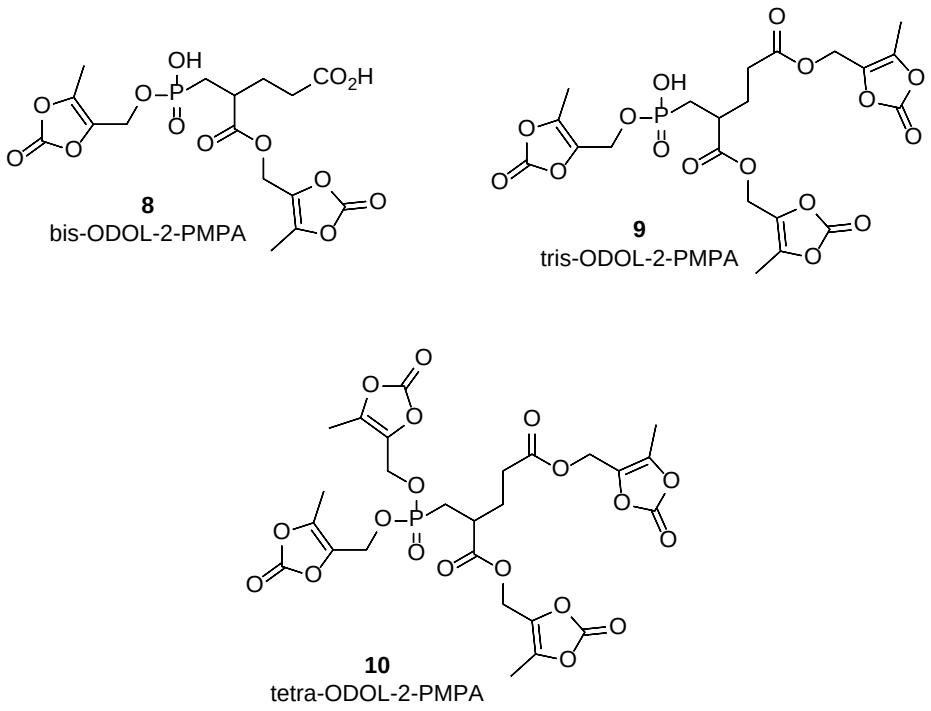
For example, **2-PMPA** prodrugs were studied and synthesized through esterification of carboxylic or phosphonic groups with pivaloyloxymethyl (POM), isopropyloxycarbonyloxymethyl (isopropyl carbonate) (POC) and (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl (ODOL) moieties, which can be functionalized by esterase enzymes [61]. Phosphonate esters with the hydrophobic POC (or POM) groups were prepared, leaving unsubstituted the α- and γ-carboxilic groups (Figure 4, **4**). These derivatives were rather unstable, with low permeability. Esterification also of the carboxylic groups enabled to enhance their chemical stability. However, they revealed to be too stable *in vivo* and minimal release of **2-PMPA** was achieved. The introduction of POC and POM groups on both the phosphonic and the α-carboxylic groups provided tris-POC-2-PMPA, tris-POM-2-PMPA, and tetra-POM-2-PMPA (Figure 4, **5-7**). Oral administration of **5** in mice for over 4 h showed >20 fold enhancement of **2-PMPA** with respect to orally administered **2-PMPA** (molar equivalent dose), showing the first example of orally bioavailable prodrugs [97].



Insert Figure 4 here

Caption: Figure 4. **2-PMPA** prodrugs.

2-PMPA prodrugs bearing ODOL groups, a FDA approved promoiety, were also prepared. Compounds containing two (**8**), three (**9**) or four (**10**) masked acidic groups (Figure 5) were synthesized from suitably substituted benzyl esters, using the different reactivity of carboxylic and phosphonate esters [96]. Their stability *in vitro*, as well as their pharmacokinetics *in vivo*, was determined in mice and dog. It was found that prodrug **10** delivered the highest **2-PMPA** levels. These results suggest that ODOL-substituted compounds offer a promising strategy to increase the oral bioavailability, opening possible space for **2-PMPA** clinical translation.



Insert Figure 5 here

Caption: Figure 5. ODOL derivatives of **2-PMPA**.

Thiol-based GCP II inhibitors

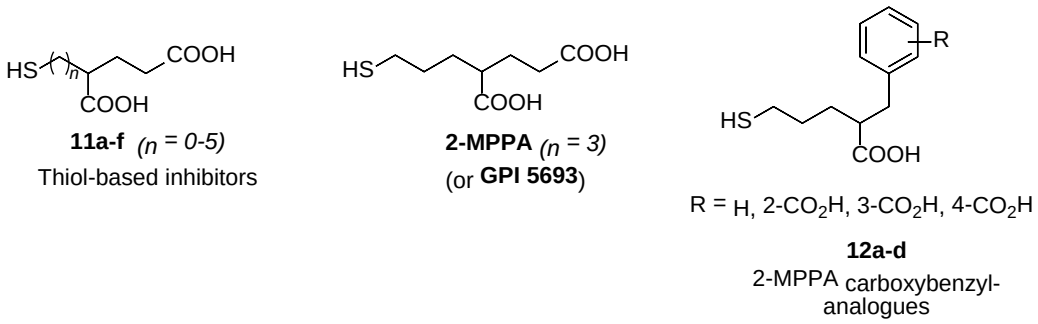
Another approach to overcome the poor oral bioavailability of the phosphorous-containing GPC II inhibitors was the preparation of molecules with a reduced polarity, bearing a less polar thiol group instead of a phosphorylated moiety. A series of 2-(thioalkyl)pentanedioic acids **11a-f** (Figure 6) were prepared and studied [98,99].

2-(Phosphonomethyl)pentanedioic acid (**2-MPPA**, Figure 6) was the first compound identified in this class of inhibitors and it proved to be efficient after oral administration in animal models of neuropathic pain, familial amyotrophic lateral sclerosis, cocaine addiction, painful and sensory diabetic neuropathy [8]. When tested in next human studies, **2-MPPA** did not cause adverse effects on CNS and it was well tolerated at plasma concentration but it did not significantly advance in clinic due to its quite low potency and concerns about the potential immune reactivity (that is common for thiol-containing drugs) [8]. Very recently, **2-MPPA** was administrated in animal models, improving working memory performance in young and aged tested rats, and it also improved performance after local infusion into the medial prefrontal cortical [100. **2-MPPA** showed also neuroprotective effects in superoxide dismutase transgenic (SOD) after induced hypoxia-ischemia [101]. Since this molecule proved to be well-tolerated, researchers suggested it could provide an important new direction for treatment of cognitive disorders associated with aging and/or inflammation and for the prevention of typical damage related to brain injury and inflammation.

Research efforts were directed towards the synthesis of more potent molecules [8], A series of derivatives of **2-MPPA** (Figure 6, **11a-f**) with a different number of methylene groups between the thiol moiety and the pentanedioic acid residue were also synthesized through different approaches, aiming to establish the optimal position of the -SH group to achieve the best affinity to GCP II [98]. The biological evaluation of the inhibitory potency against the enzyme revealed that the efficacy was dependent on the number of CH_2 groups. Indeed, 2-(3-mercaptopropyl)pentanedioic acid (**2-MPPA**, also known as **GPI 5693**, Figure 6, n = 3) was found to behave as the more active

compound in an animal model of peripheral neuropathy by oral administration (IC_{50} = 90 nM). However, none of the thiol-based compounds has been shown to be as potent as **2-PMPA**.

Both enantiomers of **2-MPPA** were also prepared and tested, showing comparable efficacy as GCP II inhibitors in the neuropathic pain model, by oral administration [87].



Insert Figure 6 here

Caption: Figure 6. Thiol-based GCP II inhibitors.

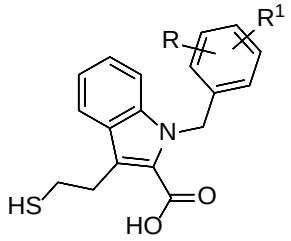
Garrido, Sanabria *et al.* investigated the functional role of **2-MPPA** with respect to its neuroprotective effects in hippocampal mossy fiber-CA3 pyramidal cell synapses [102]. Based on a mechanistic study, it is assumed that the action of **2-MPPA** in preclinical models of neurological disorders is due to a presynaptic regulation of glutamatergic neurotransmission.

In 2005 van Gerven and co-workers reported that **GPI 5693** was safe and fairly well tolerated at plasma exposures. Even if no important drug related CNS adverse events were described, minor CNS effects were observed following the highest dose level [103].

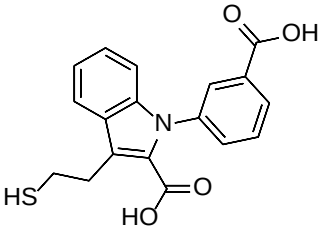
Another series of thiol-based GCP II inhibitors containing a benzyl group, in place of the carboxyethyl group of **2-MPPA**, was also synthesised (Figure 6, **12**) [104]. *In vitro* GCP II assay showed that the *meta*-substituted analogue 3-(2-carboxy-5-mercaptopentyl)benzoic acid (Figure 6, **12c**, R = 3-CO_2H) was more potent than **2-MPPA** (IC_{50} = 15 nM). On the contrary, the unsubstituted and the *ortho*-substituted derivatives (Figure 6, **12a,b**, R = H, 2-CO_2H) were less active than **2-MPPA** in inhibiting GCP II. This result was not unexpected as the removal of one carboxylate group decreases the interaction with the glutamate recognition site of GCP II. However

these results represent the first example to enhance the activity of GCP II inhibitors by modifying the glutarate moiety.

A series of *N*-substituted 3-(2-mercaptoethyl)-1*H*-indole-2-carboxylic acids **13** (Figure 7), having IC_{50} values in the range of 20-50 nM were also synthesised. Notably, at that time, derivatives **13** were the first achiral GCP II inhibitors structurally different from NAAG [105].



13a: R = R^1 = H; IC_{50} (nM): 12000 ± 5000
13b: R = 2-CO_2H, R^1 = H; IC_{50} (nM): 22 ± 21
13c: R = 3-CO_2H, R^1 = H; IC_{50} (nM): 22 ± 10
13d: R = 4-CO_2H, R^1 = H; IC_{50} (nM): 94 ± 12
13e: R = 2-Br, R^1 = 5-CO_2H; IC_{50} (nM): 54 ± 9
13f: R = 3-^{t}Bu, R^1 = 5-CO_2H; IC_{50} (nM): 34 ± 24
13g: R = 2-CO_2H, R^1 = 5-CO_2H; IC_{50} (nM): 93 ± 41



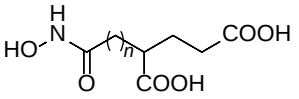
13h: IC_{50} (nM): 22 ± 4

Insert Figure 7 here

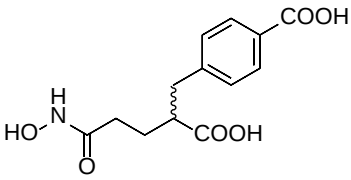
Caption: Figure 7. Structure of *N*-substituted 3-(2-mercaptoethyl)-1*H*-indole-2-carboxylic acids **13**.

Hydroxamate-based inhibitors

Other different zinc-binding compounds, as hydroxamate-based derivatives, were also investigated (Figure 8, **14a-c**). The succinyl hydroxamic acid derivative 2-(hydroxy-carbamoylmethyl)-pentanedioic acid (hydroxamate-based inhibitor (**14a**) n=1, Figure 8) showed a high GCP II inhibitor activity with an IC_{50} value of 220 nM [79]. In 2016 Barinka and coworkers reported the synthesis and the structural characterization of novel hydroxamic acid-based inhibitors (Figure 8, **14d**) [106]. These compounds contain the Zn-binding group (hydroxamic acid) bonded to the benzoic acid moiety *via* a flexible linker (2-carboxybutyl group). The racemic mixture was synthesized in eight step (27% overall yield). Chiral separation of the enantiomers, by means of a chiral column, and cleavage of the benzyl groups by hydrogenation on Pd/C led to hydroxamates (*R*)-**14d** and (*S*)-**14d** (Figure 8). Crystal structures evidenced a unique binding mode, which can explain the lack of enantiospecificity observed for the two isomers. The inhibitory properties were determined, confirming the use of such derivatives in the treatment of chronic neurological disorders, as neuropathic pain [106].



14a-c (n = 1,2,3) hydroxamate-based inhibitors



(*R*)-**14d**-hydroxamate
(*S*) **14d** hydroxamate

Insert Figure 8 here

Caption: Figure 8. Hydroxamate-based inhibitors.

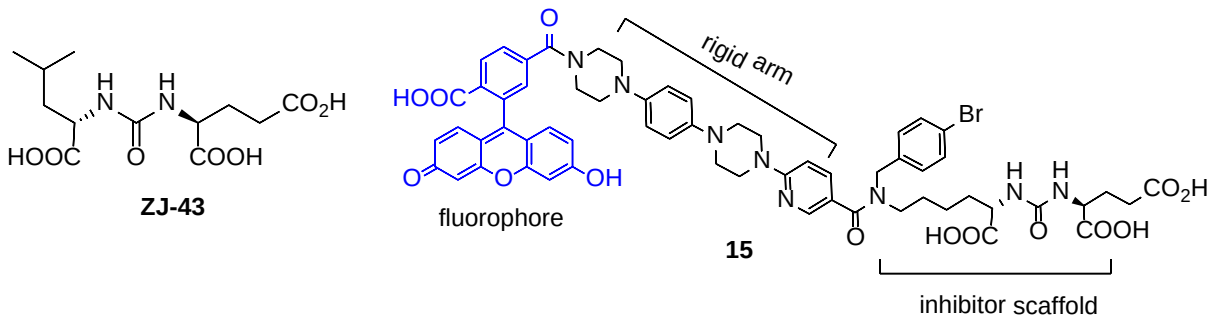
Urea-based GCP II inhibitors

Urea-based inhibitors are the third class of molecules, structurally similar to NAAG, where a urea linkage join 2 amino acids through their NH_2 groups. Urea represents here the zinc-binding group. In 2004, the urea-based NAAG analogue **ZJ-43** (Figure 9) was synthesized and its inhibitory activity on cloned human GCP II was determined using a fluorescent assay [107]. Notably, **ZJ-43** and other molecules of this group exhibited a low nM potency [8]. Remarkably, **ZJ-43** showed efficacy in various animal models of neuropathic pain, inflammatory peripheral pain, neurological disorders (*i.e.*, schizophrenia), and traumatic brain injury. On the other hand, **ZJ-43** exhibited low oral bioavailability and minimal brain penetration [8].

Several researches have been pursued in order to improve GCII inhibitors oral bioavailability and minimize the potential toxicity. In particular, the use of prodrugs has been sought after and alternative zinc-biding groups (*i.e.*, sulfamides, sulfonamides, imidazoles and other nitrogen-containing hetero-cycles) have been tested [8].

It is interesting to point out that while in case of neurological disease the employ of GCP II inhibitors increased NAAG and decreased glutamate in brain, in absence of disease the inhibition had no effect of the basal glutamate transmission. This is very important from a therapeutical point of view because it means that limiting the excess of glutamate by GCP II inhibitors may provide

neuroprotection without the undesired side effects typically observed with the use of glutamate receptors antagonists [8].



Insert Figure 9 here

Caption: Figure 9. Urea-based inhibitors.

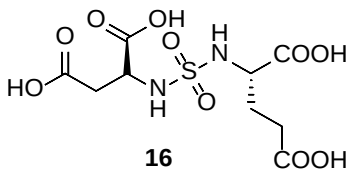
To overcome the limitations associated with poor oral bioavailability and negligible brain penetration of "traditional" GCP II inhibitors, recently, catechol-based inhibitors were developed. In particular D-DOPA offered a non-competitive mode of inhibition with an excellent pharmacokinetic profile which can be enhanced by co-administration with the DAAO inhibitor sodium benzoate, resulting in robust target engagement in the brain [108].

More recently, Šácha and coworkers developed potent urea-based GCP II inhibitors by structure-aided design [109]. The efficacy was enhanced by using a rigid linker, capable to increase the selectivity and to allow a suitable connection with different functional groups, such as a fluorophore or biotin. The replacement of the terminal PEG_{12}-biotin with the fluorescein (Figure 9, **15**) enabled a further improvement of inhibitory potency, with a K_i = 8.6 pM, with respect to the biotin-derivative (K_i = 0.11 nM).

Sulfamide-derivatives as GCP II inhibitors

In 2013 Berkman and coworkers reported a small library of sulfamide analogs of GCP II inhibitors scaffolds (Figure 10) in order to evaluate the behaviour of the sulfamide group as a zinc-binding

moiety. Compounds **16** and **17** were the most efficient inhibitors, and only the aspartyl-glutamyl sulfamide **16** showed a submicromolar activity (IC_{50} = 0.9 μM) [73].



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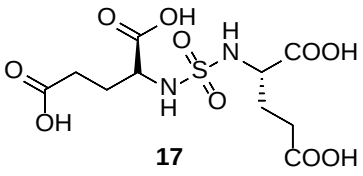

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Insert Figure 10 here

Caption: Figure 10. Sulfamides derivatives.

GCP II AND DISEASES

Cancer

Highest levels of GCP II/PSMA than normal were found in malignant tissues. In prostate cancer, the GCP II expression was found to be positively correlated with the Gleason score (cancer grade) and disease progression, increasing from the healthy tissue to androgen refractory malignancies through benign prostate hyperplasia and low grade to metastatic adenocarcinoma [3,8,48]. It is believed that, in prostate cancer, CGP II is negatively regulated by androgens and is promoted by other growth factors such as basic fibroblast growth factor, TGF (transforming growth factor) and EGF (Epidermal Growth Factor). The increased PSMA/GCP II expression in prostate cancer tissues is also related with an increased ability of cells to process folate [48].

An increased expression of GCP II was also detected in solid tumours derived from tissues that normally express the enzyme such as Schwann cells, bladder, kidney, colon and breast [3,8,69]. An increased expression of GCP II is observed in neo-vasculature of solid tumours, whereas it is absent in the vasculature of corresponding benign tissues. This suggests a possible role of GCP II in

tumour angiogenesis [3,8,48,69]. However, some significant differences were observed between different tissues tumours and between non-small cell cancers and small cell cancers [48].

GCP II/PSMA may reveal useful with theranostics purposes for these tumor types [69]. As a possible consequence of this, NAAG concentration in plasma could be a non-invasive measurement to monitor cancer progression [110].

GCP II higher expression increases folate uptake giving the cell a proliferative advantage [8,48]. Some studies highlighted that GCP II is required for carcinogenesis and cell invasion in prostate. Conversely, other studies found an inverse correlation between GCP II and prostate cancer invasiveness [8,48]. Additional investigations are required to clarify whether enzyme inhibition could block the invasiveness and growth of prostate cancer or not.

In this context, it is worth reminding the marked differences existing in the expression of GCP II between rodents and humans. The highest expression of GCP II in mice is in kidney, brain, and salivary glands, while the enzyme is almost absent in rodent prostate and small intestine. Additionally, there are no reports about the presence of GCP II in neovasculature of solid tumours in rodents [8,111]. Mouse GCP II possesses lower catalytic efficiency but similar substrate specificity compared with the human enzyme. Differences between rodents and humans also exist in the link to plasma proteins of small-molecules GCP II ligands. For all these reason, mouse GCP II could approximate human GCP II in drug development but significant differences in GCP II tissue expression need be taken into account when developing novel GCP II-based anticancer and therapeutic approaches, including targeted anticancer drug delivery systems [111].

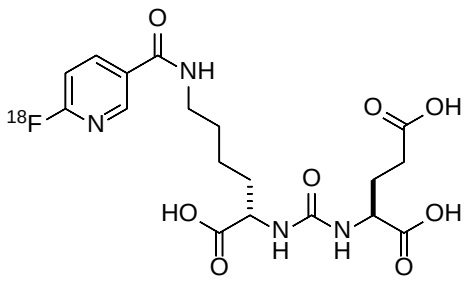
GCP II is a promising candidate both for tumours imaging/diagnosis and the delivery of toxic therapy, especially for prostatic cancer [3,8]. Monoclonal antibodies (mAb), including the ^{111}In-labelled ProstaScint^{TM} – approved by FDA as an imaging for metastatic cancer – were designed for these purposes [3]. This antibody recognizes only an intracellular epitope of GCP II/PSMA and, thus, only dying or died necrotic cells can be detected. Furthermore, ProstaScint^{TM} has to be administrated several days prior the test, with issues linked to background radioactivity [3,8]. To

overcome the limitations of ProstaScint™, antibodies that recognize the extracellular epitopes of GCP II/PSMA have been obtained. For example, J591 can be used in the imaging of both prostate and other solid tumours, owing to the presence of PSMA within the tumour vasculature [3,8]. Small radiolabelled molecules have also been used for imaging purposes. These molecules are characterized by high affinity to PSMA as well as by the possibility to be rapidly uptaken by the tumour tissue and washed-out to non-target sites [8,70].

Urea-based GCP II inhibitors have been used for imaging of prostate cancer [3,8]. ^{125}I, ^{123}I, ^{99m}Tc-labelled urea derivatives were successfully used for SPECT techniques while ^{11}C, ^{68}Ga, and ^{18}F-labelled molecules were employed for PET imaging providing high target to non-target ratio [8].

Radiolabelled **^{18}F-DCFPyL** (Figure 11) (PSMA) PET/CT was tested in women with advanced high-grade serous ovarian cancer (HGSOC). It showed higher specificity for the metastatic sites as compared to standard of care contrast-enhanced CT but detects fewer metastatic sites of disease, especially in the upper abdomen and along the gastrointestinal tract, limiting its clinical utility as a diagnostic tool in HGSOC [112].

GCP II antibodies with specific conjugated molecules can be used for cancer therapy, for example using radioactive elements as in the case of a derivative of ProstaScint™ conjugated with ^{90}Y instead of ^{111}In or ^{90}Y-J591 and ^{117}Lu-J591 [8].



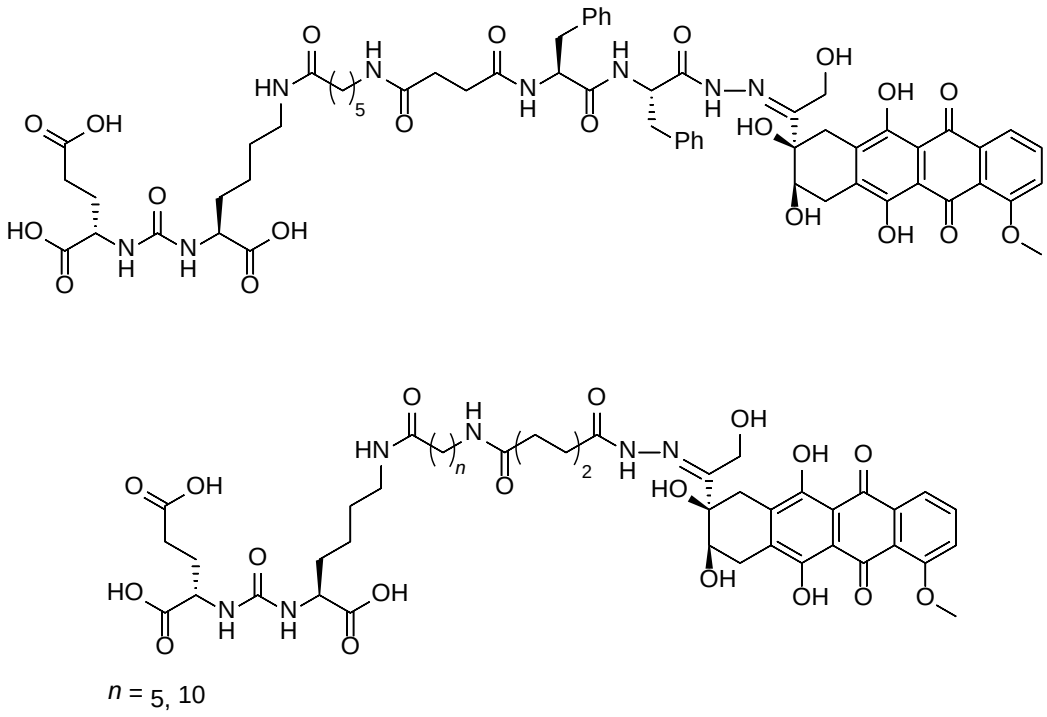
^{18}F-DCFPyL

Insert Figure 11 here

Caption: Figure 11. Radiolabelled **^{18}F-DCFPyL**.

Immunotoxins can also be conjugated to antibodies, for example this is the case of **J591** conjugated with ricin A, melitin like peptide, monomethylauristatin E and others [8]. These molecules showed promising results *in vitro* and in xenograft models, from the tumour growth inhibition to a full ablation of implanted tumours [8]. Recently PSMA-specific small-molecule carriers equipped by Doxorubicin (Dox) (Figure 12) were synthesized and tested in animal models. Preliminary results showed that the novel compounds were able to release the active substance inside cancer cells thereby providing a relatively high Dox concentration in nuclei and a relevant cytotoxic effect [113].

The issues in clinical application include the possible immunogenicity of these conjugated antibodies and the difficulties faced in producing them in a large homogeneous scale. The use of recombinant DNA technologies might help to overcome, at least partially, the problem of immunogenicity, with the obtainment of smaller, single-chain antibody fragments [8].



Insert Figure 12 here

Caption: Figure 12. Doxorubicin-derived compounds for cancer therapy.

Molecules belonging to the phosphoramidate peptidomimetic class has been radiolabelled with ^{18}F showing pseudoirreversible binding to GCP II [114]. Pseudoirreversibility is a feature of inhibitor binding that increase the internalization and that can thus be exploited for transporting therapies into PSMA-positive cells. This characteristic was not observed for urea based GCP II inhibitors [8].

All the PSMA-based imaging agents are unable to cross the blood-brain barrier because they are highly charged. Thus, although they are effective in the periphery, their use for imaging within the CNS is hampered. In order to use such derivatives in diagnosis and therapy in the brain, the removal of the tricarboxylic moiety is required [8,115].

Notably, PSMA targeting species labelled with infrared emitting fluorescent species can be used as guidance in surgeries [8,21].

Inflammatory bowel diseases

The role of GCP II in IBD (inflammatory bowel diseases) was first suggested by studies about the human disease, by genome-wide expression investigation [116]. Recently the expression of the FOLH1 gene that codes for GCP II was confirmed to be strongly upregulated in biopsies of patients with IBD [69-116-118]. FOLH1 was described as a "hub" gene that has significant correlations with over a dozen of known IBD gene biomarkers [116].

GCP II enzymatic activity has been demonstrated to be significantly increased (by 300–3000%) in both Crohn's disease and ulcerative colitis diseased patient biopsies [69,119]. The pharmacological inhibition of this upregulated activity provided therapeutic benefit in preclinical IBD models [69,119,120]. In this regard, **2-PMPA** has been proposed as a novel treatment for inflammatory bowel disease. Daily treatment with a hypotonic **2-PMPA** enema ameliorated macroscopic and microscopic symptoms of IBD in mouse model, thus highlighting the therapeutic potential of FOLH1/GCP II inhibitors for the local treatment of IBD [119,120].

Benign inflammatory states

The activity of GCP II in benign inflammatory states, including anal fistula, sarcoidosis, fasciitis, and cerebral infarction has been observed. Some expression has also been found in in Paget disease, fractures and synovitis. Although the role of the enzyme in these pathological states is poorly understood, it is interesting to target it for possible novel therapeutic strategies [69].

Male reproduction

Research on the urogenital system of aged mice highlighted that the PSMA/GCP II-deficient mouse model had increased propensity for enlarged seminal vesicles upon aging. Significant amounts of PSMA/GCP II within the mouse urogenital system were detected only in the epididymis. As the enzyme is also present in the human epididymis, these findings suggest a role of PSMA/GCP II on reproduction and provide a groundwork for further studies on humans [121].

CONCLUSIONS AND PERSPECTIVES

GCP II is currently a pharmacological and diagnostic target being pursued in the clinic for the regulation of glutamate and for imaging purposes. The inhibition of GCP II represents an interesting strategy and, although several GCP II inhibitors have been developed, some challenges remain ahead. The improvement of the oral bioavailability of GCP II inhibitors capable to penetrate the blood-brain barrier would enable a more effective treatment of neurological diseases. Indeed, although PSMA/GCP II represents a promising target for treating neurological disorders, issues in delivering drugs across the blood-brain barrier significantly hurdle its full exploration.

Additionally, the design and synthesis of specific small molecules that could discriminate between GCP II and its paralogs (*e.g.*, GCP III, whose physiological role is almost unknown but its substate specificity and pharmacologic profile are very similar to those of GCP II) would be highly desirable. In this context, more information about GCP II paralogs and orthologs in mammalian might help to better understand the role of the enzyme in healthy and diseases tissues.

Finally, the clarification of the specific function of GCP II in cancer cells would enable a more rational approach towards new potential therapeutic tools for tumour treatment.

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