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Carboxypeptidase U (TAFIa): a new drug target for fibrinolytic therapy?

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Abstract

Procarboxypeptidase U (TAFI) is a recently discovered plasma procarboxypeptidase that upon activation by thrombin or thrombin-thrombomodulin turns into a potent antifibrinolytic enzyme. Its prominent bridging function between coagulation and fibrinolysis raised the interest of many research groups and of the pharmaceutical industry. The development of CPU inhibitors as profibrinolytic agents is an attractive concept and possibilities for rational drug design will become more available in the near future due to the recently published crystal structure.

Numerous studies have been performed and many of them show beneficial effects of CPU inhibitors for the improvement of endogenous fibrinolysis in different animal sepsis and thrombosis models. CPU inhibitors combined with t-PA seem to increase the efficiency of pharmacological thrombolysis allowing lower dosing of t-PA and subsequently fewer bleeding complications.

This review will focus on recently obtained *in vivo* data and the benefits/risks of targeting CPU for the treatment of thrombotic disorders.

Introduction

The coagulation and fibrinolytic systems safeguard the patency of the vasculature and surrounding tissues. Both cascades have long been considered as separate entities but the discovery of procarboxypeptidase U (proCPU) or thrombin activatable fibrinolysis inhibitor (TAFI) greatly improved our understanding of cross regulation of both systems [1–4].

Procarboxypeptidase U is a 60 kDa metallocarboxypeptidase produced by the liver and present in plasma. By the action of thrombin, the key protease of the coagulation, this inactive zymogen is proteolytically converted to the active enzyme carboxypeptidase U (CPU). CPU potently attenuates fibrinolysis by cleaving C-terminal lysines on partially degraded fibrin, thereby interfering with efficient plasminogen activation [2–5]. A recent report claimed that the zymogen proCPU also has an intrinsic antifibrinolytic activity, however, this finding was opposed by two other groups [6–8]. Therefore activation of proCPU still provides the explicit molecular link between coagulation and fibrinolysis.

Venous and arterial thromboembolism is the largest cause of disease and death in the Western World. Therapy available today includes thrombolytics, anticoagulants and antiplatelet drugs. However, the need for parenteral application, the risk for severe bleeding complications and in the case of the oral anticoagulants the requirement for close lab

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monitoring urgently require the development of orally active antithrombotic or thrombolytic drugs that are clinically safe and need less monitoring.

Because of its prominent bridging function between coagulation and fibrinolysis, the development of CPU inhibitors as pro-fibrinolytic agents is an attractive concept. Furthermore, since the coagulation cascade is unaffected, CPU inhibition may result in fewer bleeding complications than conventional therapy.

In recent years numerous small synthetic and naturally occurring CPU inhibitors have been evaluated in animal thrombosis models and existing *in vivo* data are intriguing and call for further evaluation in humans.

CPU basic research has been extensively reviewed in the recent past [eg. 9–13]. This rather brief review will focus on recently obtained *in vivo* data and the benefits/risks of targeting CPU for the treatment of thrombotic disorders.

CPU a key modulator of the fibrinolytic threshold

Intravascular fibrinolysis is initiated when plasminogen and its activator t-PA bind to the internal lysines on the fibrin surface. Plasmin formed by the action of t-PA on plasminogen cleaves fibrin after arginine or lysine residues, generating partially degraded fibrin containing C-terminal arginine and lysine residues (initial phase of fibrinolysis). These C-terminal lysine residues participate in a multifaceted positive feedback loop. First, plasmin generation is up-regulated by the increased affinity of plasminogen for plasmin-degraded fibrin and FDPs [5,14–17]. Second, plasmin converts C-terminal lysine bound Glu^{1}-plasminogen to Lys^{78}-plasminogen, a much better substrate for t-PA [18]. Finally, C-terminal lysine residues decrease the rate of plasmin inhibition, as plasmin bound to degraded fibrin and FDPs is protected from inactivation by α_2-antiplasmin. As a result, the fibrinolytic efficiency increases dramatically (acceleration phase of fibrinolysis) [19,20].

Given the central role that C-terminal basic amino acids play in the regulation of fibrinolysis, it is not surprising that their removal from the degraded fibrin surface is also enzymatically controlled. Whereas plasmin up-regulates fibrinolysis via C-terminal lysine formation, the basic carboxypeptidase CPU downregulates fibrinolysis by removing C-terminal lysine residues from plasmin-degraded fibrin and FDPs [5,14,19,20]. The dependence of fibrinolysis on opposing processes that share components confers a threshold upon the system.

It was discovered independently by two research groups that CPU attenuates the fibrinolytic rate through a threshold dependent mechanism [21,22]. As long as CPU is present at or above a key threshold value, fibrinolysis stays in its initial phase, only to accelerate once the CPU activity decays to a level below this threshold value [21–24].

In general, thresholds are intrinsic properties of systems as a whole rather than a specific property of a single component in the system. Indeed, the critical threshold CPU concentration, defined as the concentration of CPU at which the accumulation of plasmin-catalyzed C-terminal lysine and arginine residues on fibrin is prevented, is determined by the basal steady state concentration of plasmin which on its turn is dependent on the rate of plasminogen activation (t-PA concentration) and plasmin inhibition (concentration of irreversible plasmin inhibitors) [21–24].

Leurs *et al.* [21] and Walker *et al.* [22] showed that the CPU threshold increases as the concentration of t-PA increases. With regard to the plasmin inhibitors, Walker and co-workers [24] provided evidence that the fibrinolytic process is not simply attenuated or

prolonged but can be stopped by sufficient (above the threshold) and sustained basic carboxypeptidase activity. However, this is only possible when the aggregate concentration of irreversible inhibitors is in excess of the plasminogen in the system, as is the case in plasma. Indeed, the primary plasmin inhibitor in plasma, α_2-antiplasmin, is present at only half the concentration of plasminogen (2μM) but when necessary it can act in concert with the secondary inhibitors α_2-macroglobulin (3μM) and antithrombin (5μM). In the presence of this excess of plasmin inhibitors, a sustained carboxypeptidase activity above the threshold will completely stop the fibrinolytic process. This is most probably as a result of consumption of plasminogen prior to lysis [24].

The time interval over which the CPU level will stay above the threshold is determined by the plasma proCPU concentration, the extent of proCPU activation by the coagulation cascade and most importantly by the stability of CPU [21,23]. The latter can be influenced by a naturally occurring polymorphism in the proCPU gene : the Ile-325 variant has twice the half-life of the Thr-325 variant i.e. 15 minutes versus 7 minutes at 37°C [25,26].

It remains to be determined that at the site of a thrombus, steady state concentrations of CPU are achieved that are sufficient to completely shutdown fibrinolysis. Moreover, it is still not clear whether proCPU levels and proCPU genotype influence the process [24]. This hypothesis is plausible knowing that upon activation of platelets, proCPU can be secreted from α-granules boosting local proCPU concentrations at the site of the clot [27] and that (pro)CPU can be crosslinked to fibrin by factor XIIIa [28,29]. The latter can have an important clinical relevance because proCPU and IIa come in close proximity to each other at the surface of the thrombus, leading to efficient CPU generation [30]. Moreover, an interesting hypothesis is that the interaction of CPU with fibrin can lead to a stabilisation of the carboxypeptidase, increasing its antifibrinolytic potential dramatically [21–23]. The observation that “old” thrombi are plasminogen deficient and resistant to thrombolysis further suggests that this mechanism may occur *in vivo* [24].

CPU: a new drug target?

Drug design: the need for a crystal structure

Having a crystal structure at your disposal could be of great benefit for efficient rational drug design. However, due to the very low solubility of the enzyme, the glycosylation extent of the zymogen, and the pronounced thermal instability of the active enzyme, attempts to crystallize (pro)CPU remained unsuccessful. Mutagenesis studies of Knecht *et al.* [23] and Ceresa & coworkers [31,32] showed that limited mutagenesis in the protein-covering β-sheet 9 and α-helix 11 (residues 297–335) can dramatically increase the stability of CPU mostly with conserved wild-type characteristics. These mutants were a first step to understand the mechanism of inactivation of CPU and are potent tools to facilitate structural characterization. Most recently the crystal structure of proCPU was solved by Marx *et al.* [33]. ProCPU was expressed in a cell line lacking *N*-acetylglucosaminoyltransferase I yielding a proCPU recombinant form with homogenous *N*-linked glycans. This trick made it possible to grow properly diffracting crystals and to solve the proCPU structure [33]. The crystal structure explains the proCPU auto-regulation mechanism. Briefly, in the zymogen the activation peptide not only covers the active site but also stabilizes a dynamic segment of the enzyme moiety (residues 296–350). Proteolytic activation results in release of the activation peptide and concomitant increase in dynamic segment motility. The increased dynamics lead to conformational changes that disrupt the catalytic site [33]. Using this model we understand now that binding of reversible CPU inhibitors (see below) can stabilize CPU activity by reducing the mobility of the dynamic segment. The structural data of Marx were confirmed by two publications of another group [34,35].

CPU inhibitors: an overview

A specific inhibition of metallocarboxypeptidase activity can be achieved by agents such as o-phenantroline and EDTA that chelate the essential Zn ion in its active site [1,36,37]. CPU is also sensitive to dithiothreitol and 2-mercaptoethanol because of the presence of disulphide bridges in the active moiety [1,36,37]. The organic inhibitors 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MERGETPA) and guanidinoethylmercaptosuccinic acid (GEMSA) are the most widely used inhibitors for both *in vitro* and *in vivo* studies with Ki values in the order of mM to μM [12]. However, both compounds have two major drawbacks from a drug discovery point of view. Firstly, both compounds are potent inhibitors of carboxypeptidase N (CPN). CPN is believed to have an important function in plasma as an inactivator of anaphylatoxins and in the processing of peptide hormones [38,39]. Consequently, selectivity towards CPN is an important parameter to consider during optimisation. Secondly, both compounds are extremely polar resulting in a low oral bioavailability [12]. Major efforts have been made to obtain more potent and selective drugs with favourable pharmacokinetic properties. An overview of synthetic CPU inhibitors is presented in Table 1. Although a high selectivity towards CPN is obtained by those inhibitors, obtaining selectivity towards the digestive basic carboxypeptidase, CPB, seems less straightforward [12 and references of Table 1]. However, little risk is anticipated for transient inhibition of pancreatic CPB since inhibition of this carboxypeptidase should not lead to intestinal malabsorption due to the presence of alternate active proteases available for digestion.

Apart from the synthetic inhibitors, protein inhibitors also have been described. The most familiar one is potato tuber carboxypeptidase inhibitor (PTCI), a 39 amino acid protein that competitively inhibits CPU with a Ki in the nM range [40]; others are the 66 residue leech carboxypeptidase inhibitor (LCI) found in *Hirudo medicinalis* [41] and the recently isolated tick carboxypeptidase inhibitor (TCI) from the soft tick *Rhipicephalus bursa* [42]. A major advantage of this kind of inhibitors is the high selectivity for CPU.

The promising results of CPU inhibitors have been somewhat overshadowed by the observation that in *in vitro* experiments several competitive inhibitors such as GEMSA and PTCI show a biphasic effect, prolonging clot lysis at low concentrations and enhancing lysis at high concentrations [44,62]. This can be explained by the notion that free and (inhibitor) bound CPU stay in equilibrium. While the free CPU inactivates irreversibly, the bound form, protected against spontaneous inactivation (due to decreased dynamic segment mobility), is released to replenish the free pool, so as to maintain the equilibrium [44,62]. As long as the free CPU concentration stays above the t-PA dependent threshold value, fibrinolysis will stay in its initial phase. Understandably this stabilising effect of CPU inhibitors has raised concerns, but one should keep in mind that *in vitro* observations are not always mirrored in the *in vivo* situations. Indeed, thus far *in vivo* data do not substantiate the *in vitro* observed paradoxical behaviour of competitive CPU inhibitors. However, this phenomenon needs to be further investigated, especially in *in vivo* animal models and in the clinic. Of note a minor or absent antifibrinolytic effect was observed at low concentrations of TCI and LCI [41,42]. These inhibitors show a 10-fold higher potency than PTCI. Moreover, their larger size (nearly twice of PTCI) and additional or more efficient contacts with the enzyme result in a higher stability of the enzyme inhibitor complex. This most probably results in the less apparent antifibrinolytic effects at low inhibitor concentrations [41,42]. With the available crystal structure, more efficient drug design will reveal potent non stabilising small synthetic CPU inhibitors in the near future.

A final pharmacological approach to target CPU is by hampering its generation from proCPU. Potential physiologic activators include thrombin, thrombin-thrombomodulin (T-

TM) and plasmin. Several monoclonal antibodies have been described that inhibit the conversion of proCPU into CPU by T-Tm and plasmin [63]. Although activation of proCPU by thrombin or plasmin involves the same cleavage site, Arg92, Hillmayer *et al.* recently demonstrated that some of these antibodies can discriminate between plasmin or T/TM mediated activation, providing strong evidence that (i) those MA do not bind to the cleavage site and (ii) that different binding residues in proCPU exist for thrombin/plasmin [64]. Indeed, MA that inhibit exclusively the activation of proCPU by thrombin-thrombomodulin bind to Gly^{66} and MA that inhibits the activation by both T/TM and plasmin bind to Val^{41} [64]. Those MA are interesting tools to investigate physiological activation of proCPU *in vivo*. Recently mAbTAFI/TM#16, a MA antibody that interferes with the T/TM activation has been tested *in vivo* in a baboon model of *E.coli* induced sepsis [65]. In this model T-TM appeared to be the predominant activator of proCPU *in vivo* [65]. Of interest, those MA do not cross react with CPN or CPB theoretically favouring their therapeutic use, although one should consider the need for parenteral administration and their expensive nature.

Clinical utility of CPU inhibition or a role for CPU in vivo?

Data from knockout mice

Targeted gene disruption of the proCPU gene did not result in an overt phenotype [66]. ProCPU knockout mice are indistinguishable from their WT littermates concerning survival, development and fertility. Additionally, no major differences were revealed between KO and WT mice in hemostatic analyses including bleeding times following tail transaction [66].

Unexpectedly, Nagashima *et al*. did not observe significant differences between KO and WT mice in several thrombosis models including thrombin-induced thromboembolism, factor X coagulant protein-induced thromboembolism, endotoxin-induced thromboembolism, or time to vascular occlusion in phytochemical-induced vascular injury [66]. This raised questions about the *in vivo* significance of the proCPU/CPU system. However, by backcrossing proCPU deficient mice to a heterozygous plasminogen deficient background, a role for proCPU was demonstrated in models of pulmonary embolism and peritoneal inflammation indicating that proCPU can modulate the *in vivo* functions of plasmin(ogen) in fibrinolysis and cell migration [67]. Recently, new data on proCPU knock out mice with a normal plasminogen status have been published showing a significant *in vivo* role of CPU [68]. Wang *et al.* demonstrated that proCPU deficiency protects mice from 3.5% ferric chloride-induced vena cava thrombosis: a 34% reduction in thrombus weight occurred in $proCPU^{-/-}$ but not in $proCPU^{+/-}$ mice (3% reduction) compared to wild type littermates. However, in a more severe injury model (5% $FeCl_3$) the difference in thrombus weight between $proCPU^{-/-}$ and $proCPU^{+/+}$ mice was not significant anymore [68]. Mao *et al.* [69] demonstrated that, *in vivo*, in a batroxobin-induced pulmonary embolism model, proCPU knock out mice (with normal plasminogen status) displayed a lower retention of fibrin in the lungs than did WT littermates, thus linking proCPU deficiency with enhanced endogenous fibrinolysis

Improvement of endogenous fibrinolysis

Several studies have been conducted to evaluate whether a CPU inhibitor alone improves endogenous thrombolysis, albeit with contradictory results. The *in vivo* profibrinolytic efficiency of a CPU inhibitor alone depends on the type of thrombosis model and the studied animal species, the type of inhibitor and whether this inhibitor is administered before or after thrombus induction. Minnema *et al.* [70] showed that incorporation of anti-factor XI antibodies or PTCI in jugular vein thrombi resulted in both cases in an almost twofold increase in endogenous thrombolysis compared with controls, whereas inhibition of both

factor XI and CPU activity had no additional effect. However, in two other studies, infusion of PTCI *after* thrombus formation in the isolated segment of the jugular vein [71], and in the abdominal aorta in rabbits [49], did not enhance endogenous thrombolysis. The latter studies suggest that administration of a CPU inhibitor alone is not efficient after thrombus formation. However, Muto *et al.* [47] and Suzuki *et al.* [61] demonstrated that tissue factor-induced microthrombi can be efficiently lysed by systemic injection of MERGETPA, PTCI or EF6265 alone even after formation of thrombi. Also Hashimoto & coworkers demonstrated enhancement of endogenous fibrinolysis on addition of PTCI or argatroban (a direct IIa inhibitor) after thrombus formation in an arterial thrombolysis model using rat mesenteric arterioles [72]. Interestingly, Wang *et al.* [50] elaborated on the importance of the magnitude of the thrombotic stimulus to see an overt effect of a CPU inhibitor alone. Indeed, PTCI, administered after thrombus formation, significantly inhibits murine thrombosis in the absence of exogenously administered t-PA using a model of ferric chloride (3.5 %)-induced vena cava thrombosis. However, in a more severe injury model (5% $FeCl_3$) the effect of PTCI was not significant anymore. PTCI alone had also no effect on 3.5% $FeCl_3$ –induced carotid artery thrombosis [50]. In a rat DIC model it has been demonstrated that inhibition of CPU by BX528 attenuated LPS induced resistance to endogenous fibrinolysis [58,59]. Most recently Muto *et al.* [60] showed in a rat endotoxemia and sepsis model that administration of EF6265 1 hour after the intravenous injection of lipopolysaccharide (LPS) and Pseudomonas *aeruginosa*, respectively, results in decreased deposition of fibrin in the kidney and liver without significant changes in platelet and fibrinogen concentrations. Moreover, this compound also significantly decreased levels of plasma lactate dehydrogenase and aspartate aminotransferase, markers of organ dysfunction [60].

Recently, a phase II, single-blind, multicentre study was presented investigating the effect of the novel CPU inhibitor AZD9684 in pulmonary embolism [56]. Fifty-eight patients with confirmed PE were randomized to receive AZD9684 or placebo, on top of once-daily dalteparin for 5–7 days. In the patient group receiving AZD9684, fibrinolysis biomarkers in plasma were higher and sustained for a longer period of time, implying that inhibition of CPU by AZD9684 stimulates endogenous fibrinolysis. Moreover, lung deficiency scintigraphy scores improved over the treatment period. In addition, no difference in the occurrence of adverse effects was seen between both treatment groups.

In conclusion, to date there is still a high need for more *in vivo* data to draw more definite conclusions about the efficiency of the administration of a CPU inhibitor alone in venous and arterial thrombosis models.

Adjuvants for thrombolytic therapy

Thrombolysis consists of the pharmacological dissolution of a blood clot by intravenous infusion of plasminogen activators that activate the fibrinolytic system. The clinical benefits of thrombolytic therapy in patients with acute myocardial infarction and ischemic stroke are well documented. However, all available thrombolytic agents still have significant shortcomings, including the need for large therapeutic doses, limited fibrin specificity and, most importantly, significant associated bleeding tendency and reocclusion [73]. For example, treatment of acute myocardial infarction with thrombolytic therapy is associated with a failure to lyse the clot in 15 to 50% of coronary thrombi, 10–25% of successfully recanalised vessels re-occlude and up to 2% of treated patients suffer from major bleeding complications [74,75].

Tremendous benefit could be expected from adjunctive therapy that potentiates the t-PA mediated thrombolytic effect and that consequently reduces the dose of plasminogen activators, hereby potentially reducing unfavourable side effects.

It is well known that thrombolytic therapy by t-PA or other thrombolytic agents induces a local procoagulant state. The observation of increased plasma fibrinopeptide A following such therapy is consistent with release of active IIa in the vasculature, and could help explain the observations of rethrombosis following successful thrombolysis [30,76]. Moreover, based on studies in both experimental animals and patients, it appears that IIa inhibitors accelerate thrombolysis and improve clinical outcome [76,77].

Mattsson *et al*. [78] demonstrated local *in vivo* proCPU activation in the coronaries during thrombolytic treatment in a dog model of coronary artery thrombosis. This CPU generation could be inhibited with a direct, small molecule IIa inhibitor (melagatran). This suggests that the profibrinolytic effects of direct IIa inhibitors may, at least partly, be due to an inhibition of IIa mediated activation of proCPU [78]. Indeed, the shortening in lysis time when rt-PA was combined with melagatran is comparable to the reduced lysis time seen on addition of MERGETPA to rt-PA in the same model [48]. We recently showed a similar CPU generation in patients with acute ischemic stroke being treated with intra-venous rt-PA or local intra-arterial urokinase administration [79] and showed that this CPU generation decreases the efficacy of thrombolytic therapy in these patients [80]. Indeed, the peak CPU activity during thrombolysis was associated with the evolution of the clinical deficit and the achieved recanalisation and the amount of proCPU consumption during thrombolysis was related to the risk of intracranial hemorrhage, mortality and final infarct volume [80]. Although these data need confirmation in larger studies it is the first step towards investigating the utility of CPU inhibitors as adjuvant treatment with tPA or urokinase in acute ischemic stroke in humans.

In recent years consistent data have been obtained in animal studies on the utility of CPU inhibitors as adjuvant therapy with thrombolytics. Klement *et al*. investigated the impact of a CPU inhibitor (PTCI) on t-PA induced clot lysis in a rabbit model of arterial thrombolysis [49]. They found that when the inhibitor was administered along with t-PA, numerous parameters associated with the efficiency of thrombolysis were enhanced. The time to reperfusion was reduced by an approximate factor of three. Vessel patency was similarly improved [49]. Similar studies were carried out by Nagashima *et al*. [71] in a rabbit model of venous thrombolysis, where the impact of PTCI on t-PA mediated clot lysis was determined. When PTCI was administered along with t-PA, the clot weight was reduced to approximately half of the control weight. t-PA alone at the same dose reduced clot weight only 26%. This combined effect of t-PA and PTCI could be obtained with three times the dose of t-PA in the absence of PTCI. Recently Wang *et al.* [58,59] demonstrated that administration of BX 528 enhances the thrombolytic effects of low dose t-PA in a dog model of femoral artery thrombosis induced by 10 % $FeCl_3$ injury, a rat model of femoral artery thrombosis induced by photochemical injury and a rabbit *ex vivo* model of jugular vein thrombolysis.

Safety of CPU inhibition

One of the key concerns in antithrombotic therapy is the bleeding risk. Will the pharmacological inhibition of CPU result in an increased bleeding risk? It has been demonstrated that proCPU deficiency did not cause spontaneous bleeding or alterations in the bleeding time [66]. Moreover, the use of a CPU inhibitor in many *in vivo* animal models was not associated with an increased bleeding risk either when the inhibitor was used alone or in association with a low dose t-PA. There is only one study that shows a bleeding liability for PTCI [50]. The average bleeding time prolongation (mouse tail bleeding model) achieved at the top dose of PTCI was similar to that of aspirin (30 mg/kg); however none of the mice dosed with PTCI showed off-scale bleeding. In contrast, the 30 mg/kg dose of aspirin resulted in 36% of the mice-bleeding off-scale [50].

Important to realise is that besides its antifibrinolytic function, carboxypeptidase U may also play a role in inflammation [81,82]. Indeed, bradykinin and the complement factors C3a and C5a are well known CPU substrates [83–85] and recent studies show that proCPU plays a role in regulating complement-mediated vascular inflammation. It remains to be investigated whether CPU inhibitors can be used as profibrinolytic drugs in pathological conditions like atherosclerosis and arterial thrombosis where inflammation plays a pivotal role. A recent case control study reported that low plasma levels of proCPU are associated with increased risk of a first myocardial infarction [86]. These data, however, should be confirmed in a prospective study design where it should be investigated whether the low proCPU levels are causally linked to an increased risk of myocardial infarction or whether they are provoked by an increased activation to CPU due to a hypercoagulative state in these patients.

In the past years interesting animal studies have been published about the role of CPU in inflammation. It has been demonstrated that proCPU is a positive acute phase protein in mice as administration of bacterial lipopolysaccharide to these animals resulted in marked increase in hepatic CPB2 mRNA abundance and plasma proCPU protein concentrations [87]. However when interpreting these data one should keep in mind the important differences in the sequences of the proCPU promoters from human and mouse. Indeed unlike the mouse promoter, the human promoter is not induced by inflammatory cytokines [10]. In line with this observation, proCPU levels decreased significantly upon administration of low dose LPS to human volunteers whereas CRP increased more than 100-fold [88]. Also low proCPU plasma levels have been reported in patients with sepsis [89].

ProCPU knockout mice were protected from liver necrosis after intra peritoneal injection with Escherichia *coli* [90] and in proCPU/plasminogen double knock-out mice, the migration of leucocytes towards the peritoneum was increased in the proCPU-deficient animal models compared to the wild-types showing the importance of proCPU in plasminogen-dependent cell migration *in vivo* [83]. Moreover, it was shown that proCPU deficient mice have a wound-healing problem which may be related to this cell migration process [91]. These *in vivo* data suggest a role for CPU in inflammation, but at the same time call for further investigation.

Conclusions

Twenty years after its discovery, CPU has turned into a hot topic in the field of thrombosis and haemostasis. This carboxypeptidase is currently recognized as an important molecular link between coagulation and fibrinolysis, and selective CPU inhibitors have been designed and tested in several thrombosis models. Animal studies show improved endogenous fibrinolysis and an increased efficiency of t-PA mediated thrombolysis upon inhibition of the (pro)CPU system. Recently, a phase II, single-blinded, multicentre study in patients with pulmonary embolism also showed enhancement of endogenous fibrinolysis upon administration of a selective CPU inhibitor. These intriguing *in vivo* data call for further evaluation in humans.

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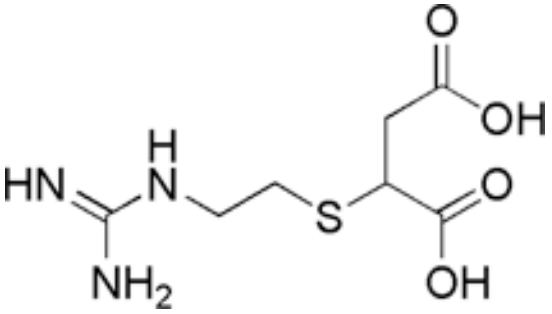
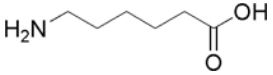
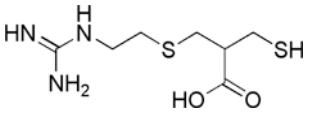
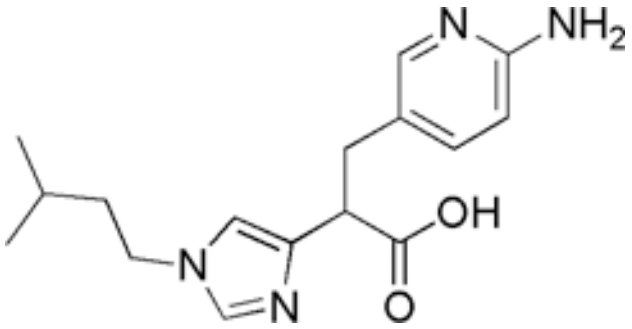
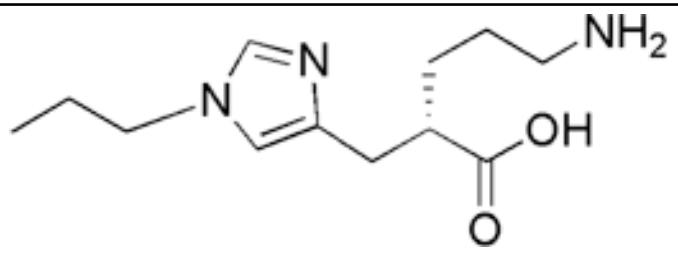
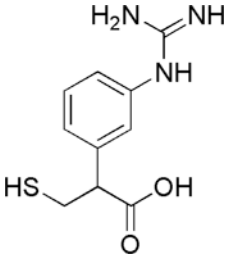
Table 1

Small synthetic inhibitors and naturally occurring metallocarboxypeptidase inhibitors

Compound	Structure	Inhibition constants	Specificity vs. human CPN	Specificity vs. human CPB	Model	Ref
guanidinoethyl-mercaptosuccinic acid (GEMSA)		$K_i = 10^{-6} – 10^{-4}$	0,025-fold	0,011-fold	not available	37,43, 44,45
ε-amino caproic acid (ε-ACA)		$K_i = 10^{-3} – 10^{-2}$	0,8-fold	0,9-fold	not available	43,45, 46
DL-2-mercaptomethyl-3-guanidinoethyl-thiopropanoic acid (MERGETPA)		$K_i = 10^{-7} – 10^{-6}$	0,009-fold	not available	- TF-induced renal microthrombi model - dog model of coronary arterial thrombosis	37,45, 47,48
potato tuber carboxypeptidase inhibitor (PTCI)	39 amino acid polypeptide	$K_i = 10^{-10} – 10^{-9}$	> 25 000-fold	0,5-fold	- rabbit arterial thrombolysis model - murine model of ferric chloride-induced vena cava thrombosis - TF-induced renal microthrombi model	40,47, 49,50

Compound	Structure	Inhibition constants	Specificity vs. human CPN	Specificity vs. human CPB	Model	Ref
leech carboxypeptidase inhibitor (LCI)	66 amino acid polypeptide	$K_i = 10^{-9}$	not available	not available	not available	41
tick carboxypeptidase inhibitor (TCI)	75 amino acid cysteine-rich polypeptide	$K_i = 10^{-9}$	> 100 000-fold	1,1-fold	not available	42
SAR-104772	not available	not available	not available	not available	not available	51
Compound (−)-8		IC_{50} = 2 nM	> 25 000-fold	1,35-fold	African green monkey model of vascular injury	51–53
UK-396,082		$K_i = 10^{-8} – 10^{-9}$	> 1000-fold	not available	rabbit model of venous thrombosis	51,54
Compound (−)-24		IC_{50} = 3 nM	600-fold	2-fold	rabbit model of venous thrombosis	51,55
AZD-9684	not available	not available	not available	not available	phase II, single-blind, multicentre study on acute pulmonary embolism patients	51, 56

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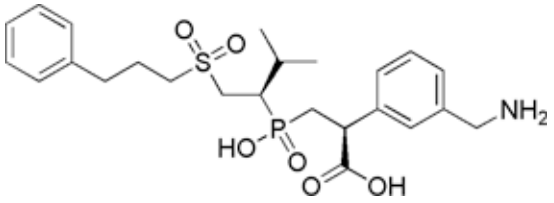
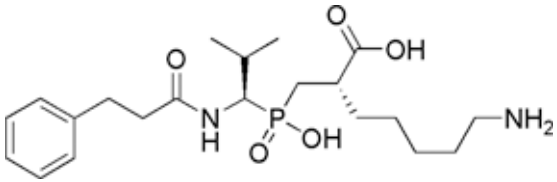
Compound	Structure	Inhibition constants	Specificity vs. human CPN	Specificity vs. human CPB	Model	Ref
BX 528		$K_i = 10^{-9}$	> 35 000-fold	12-fold	four different animal models (rat, dog, rabbit) of thrombosis employing different thrombogenis stimuli ($FeCl_3$, laser, ex vivo and batroxobin) in different tissues (artery, vein and lung microcirculation)	51,57
EF6265		$K_i = 10^{-8}$	> 750-fold	not available	sepsis-induced organ dysfunction in rats	51,60, 61