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# The emerging role of dipeptidyl peptidase 3 in pathophysiology

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Dipeptidyl peptidase 3 (DPP3), a zinc-dependent aminopeptidase, is a highly conserved enzyme among higher animals. The enzyme cleaves dipeptides from the N-terminus of tetra- to decapeptides, thereby taking part in activation as well as degradation of signalling peptides critical in physiological and pathological processes such as blood pressure regulation, nociception, inflammation and cancer. Besides its catalytic activity, DPP3 moonlights as a regulator of the cellular oxidative stress response pathway, e.g., the Keap1-Nrf2 mediated antioxidative response. The enzyme is also recognized as a key modulator of the renin-angiotensin system. Recently, DPP3 has been attracting growing attention within the scientific community, which has significantly augmented our knowledge of its physiological relevance. Herein, we review recent advances in our understanding of the structure and catalytic activity of DPP3, with a focus on attributing its molecular architecture and catalytic mechanism to its wide-ranging biological functions. We further highlight recent intriguing reports that implicate a broader role for DPP3 as a valuable biomarker in cardiovascular and renal pathologies and furthermore discuss its potential as a promising drug target.

## Introduction

Dipeptidyl peptidases (DPPs) are enzymes classified as EC3.4.14 in the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology system of enzyme nomenclature [1]. Generally,

**Abbreviations**
ACE, angiotensin converting enzyme; ANG, angiotensin; ARE, antioxidant response element; Arg-Arg-βNA, Arg-Arg-β-naphthylamide; $AT_1R$, angiotensin II receptor type 1; btDPP3, bacterial dipeptidyl peptidase; CDK1, cyclin-dependent kinase 1; cDPP3, circulating dipeptidyl peptidase 3; CRC, colorectal cancer; CRVS, catecholamine-resistant vasodilatory shock; DPP, dipeptidyl peptidase; $ER^+/-$, estrogen receptor positive/negative; hDPP3, human dipeptidyl peptidase; $IC_{50}$, half maximal inhibitory concentration; $k_{cat}$, catalytic rate; Keap1, kelch like ECH associated protein 1; Nrf2, nuclear factor-erythroid factor 2-related factor 2; OGD/R, oxygen-glucose deprivation/reoxygenation; PCZ, procizumab; $PR^+/-$, progesterone receptor positive/negative; RAS, renin-angiotensin system; ROS, reactive oxygen species; VSCM, vascular smooth muscle cells; yDPP3, yeast dipeptidyl peptidase.

the family of DPPs is represented by eight members in mammalian species, DPP1 (cathepsin-C), DPP2 (DPP7), DPP3, DPP4 (CD26), DPP6, DPP8, DPP9 and DPP10 [1], and in addition, two family members, DPP5 and DPP11, exclusively found in bacteria [1,2]. DPPs are involved in almost every aspect of life encompassing protein maturation and degradation, cell cycle regulation, processing and degradation of bioactive peptides as well as the defence against oxidative stress [1,3–7]. Several studies have implicated DPPs in pathophysiological processes; however, the exact nature and mechanisms remain to be explored [8–19].

Dipeptidyl peptidase 3 (DPP3, EC3.4.14.4) gained its name from being the third discovered enzyme from the dipeptidyl peptidase family. DPP3 was first isolated from the bovine anterior pituitary gland in 1967 [20] and appeared under different names such as dipeptidyl arylamidase III [20], dipeptidylaminopeptidase III [21,22] and enkephalinase B [23]. It was later isolated from various sources including microorganisms, parasites, insects and various mammalian tissues and cells, including humans (Table 1). Notably, *Dpp3* is ubiquitously and abundantly expressed in human cells leading to the inclusion of DPP3 into the central proteome [24,25].

DPP3 is a zinc-dependent aminopeptidase that specifically cleaves dipeptides at the N-terminus of oligopeptides ranging from four to ten amino acid residues [26]. It binds one zinc ion per subunit and has a molecular mass around 80-85 kDa. The protein adoptes a bi-lobed structure featuring two conserved sequence motifs (HEXXGH and EEXRAE/D) both of which are important for the coordination of the $Zn^{2+}$ ion in the active site of the protein [26].

DPP3 is primarily a cytosolic protein [22,27–30] although some studies suggested a potential membrane localization [31–35]. The peptidase was found to be associated with important physiological and pathological pathways in mammals, with accumulating evidence for its involvement in protein turnover [36,37], oxidative stress [8,33,38–42], pain modulation, carcinogenesis [10,11,18,19], inflammation [8,12–14,27] as well as blood pressure regulation and the renin-angiotensin system [43–47].

**Table 1.** Detection and isolation of DPP3 from various organisms.

| Species | Tissues | References |
|---|---|---|
| Microorganisms | | |
| *Saccharomyces cerevisiae* | | [107] |
| *Dicotyostelium discoideum* | | [108,109] |
| *Pediococcus acidilactici* | | [110] |
| Parasites | | |
| *Schistosoma japonicum* | | [111] |
| *Schistosoma mansoni* | | [111] |
| *Entamoeba histolytica* | | [112] |
| *Drosophila melanogaster* | | [33] |
| *Blaberus craniifer* | | [113] |
| Mammals | | |
| *Rattus rattus* | Brain, heart, intestine, kidney, liver, lung, pancreas, salivary gland, muscle, spleen, testes, thymus | [21,28,50,114] |
| *Ovis aries* | Brain | [53] |
| *Bos taurus* | Lens | [22,49] |
| *Mus musculus* | Lens | [115] |
| *Oryctolagus cuniculus* | Erythrocytes | [116] |
| *Sus scrofa domesticus* | Spleen, muscle | [75] |
| Sciuridae | Liver | [117] |
| *Homo sapiens* | Blood cells | [12,36,118,119] |
| | HepG2 cells | [120] |
| | Lens | [121] |
| | Placenta | [30] |
| | Muscle | [122] |
| | Renal epithelial cells | [53] |

## Localization of DPP3

DPP3 is mainly described as a soluble, cytosolic protein in mammals [22,28–30]. Proteome analysis of mouse pluripotent stem cells additionally assigned DPP3 as a cytosolic protein with high confidence [48]. However, membrane-associated activity has been described in *Pediococcus acidlactici*, *Drosophila melanogaster* [33], calf brain [49] and several rat tissues [50]. Moreover, the translocation of DPP3 into the nucleus was reported under the conditions of oxidative stress [51,52]. In view of the conflicting evidence, further research into the localization of DPP3 will be necessary.

DPP3 has also been found extracellularly in the cerebrospinal fluid of sheep and human [41,53], human seminal plasma [54] and retroplacental serum [30]. The presence and activity of DPP3 in human plasma of healthy subjects has been recently reported [45]. Deniau et al. [47] and Takagi et al. [46] also described elevated circulating DPP3 (cDPP3) activity in patients with cardiogenic shock. In addition, it was found that elevated cDPP3 levels are associated with a poor outcome in patients suffering from sepsis [55,56]. Recent reports have further highlighted the role of cDPP3 in cardio-renal disease progression, which will be discussed in detail below [57,58].

## Structure and catalytic mechanism of DPP3

To date, the crystal structures of human (hDPP3), yeast (yDPP3) and bacterial (btDPP3) DPP3 have

been reported, providing insight into its mode of substrate binding as well as the catalytic mechanism of the enzyme. Although the sequence identities of btDPP3 and yDPP3 compared to hDPP3 are quite low (17–21% and 36%, respectively), the overall structure of all reported DPP3 homologs are very similar [59–61].

DPP3 is composed of two domains that are connected by a hinge region. The two domains are separated by a wide cleft that forms the peptide binding site (Fig. 1A). While the upper lobe is mostly helical, the lower domain consists of a mix of α- and β-secondary structures with a five-stranded β-barrel core (two-stranded in case of btDPP3) [59–61].

Upon the formation of the enzyme-peptide complex the two domains move towards each other, leading to the closure of the cleft and consequently to the burying of the bound peptide [60] (Fig. 1B). Amino acids 409-420 were identified as mechanical hinge and they are evolutionarily conserved, particularly at the N-terminal part of the region where secondary structures are developed [60]. The C-terminus of the hinge section forms a pronounced U-shape, stabilized by hydrogen bonds to Lys-670. Lys-670 is a highly conserved residue amongst the known DPP3 homologs with the exception of yDPP3, where an arginine occupies this position, presumably engaging in similar interactions [60]. Upon binding of tynorphin (Val-Val-Tyr-Pro-Trp), Lys-670 interacts with the C-terminus of the peptide, moving away from its previous position, and thus, presumably inducing the conformational change in the hinge region [60]. Upper and lower domains move as rigid bodies to achieve a 60° rotation (28° in btDPP3) of one lobe relative to the other [60,61]. This domain movement was suggested to be the rate-determining step of the catalytic reaction [66]. The magnitude of the conformational change that leads to the closed enzyme-substrate complex is uncommon due to the involved entropic costs [60,67]. However, the studies of Bezerra et al. [60] revealed that the binding of tynorphin to hDPP3 is entropically favoured by the release of all or at least most of the 60 ordered water molecules from the binding site.

All described DPP3s contain the two unique zinc binding motifs that are characteristic for the metallopeptidase family M49 [60]. Both motifs are part of the

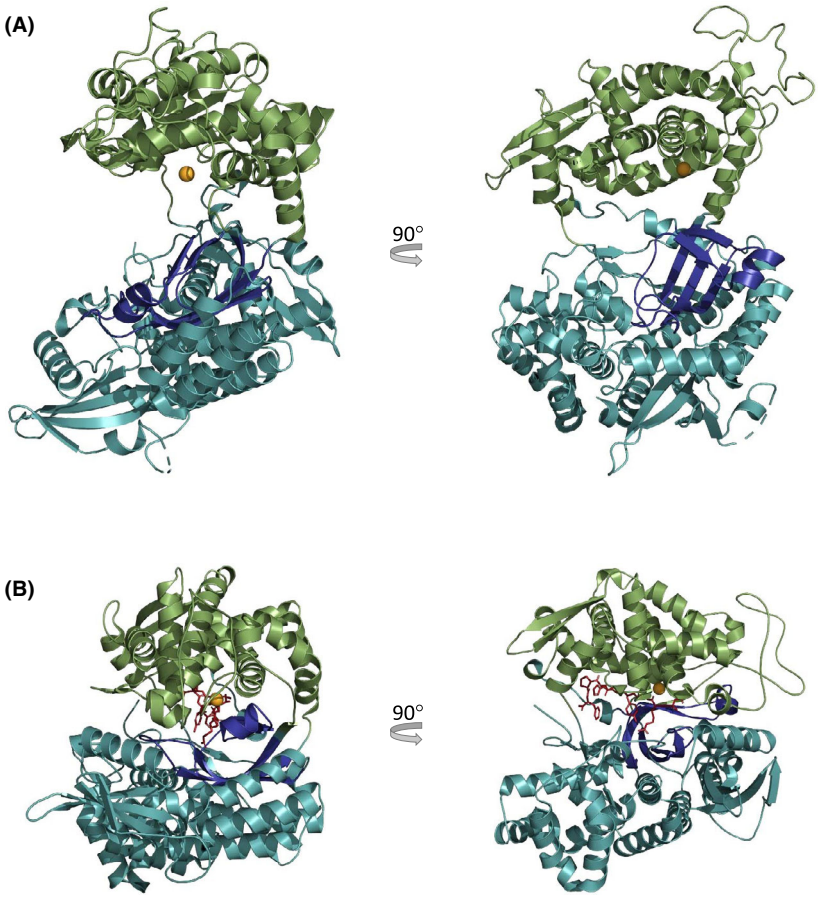


**Fig. 1.** Overall crystal structure of hDPP3. (A) Two views of the unliganded hDPP3 structure in its open conformation. The upper lobe that contains the zinc binding motifs is shown in green. The lower lobe is depicted in light blue with the five-stranded β-barrel being highlighted in dark blue. The zinc ion is shown in orange. (B) Two views of the structure of hDPP3 in complex. Figures were created in PyMol with the PDB entries 3FVY and 5E2Q. The zinc ion was superposed from PDB entry 5E33.

mostly helical upper domain, and together, they coordinate a zinc ion per subunit to the 'roof' of the cleft [62,63]. The histidine residues of the conserved HEXXGH motif coordinate the zinc ion while the glutamic acid acts as a catalytic base [64].

On the other hand, the EEXR(K)AE(D) motif is found 22-55 amino acids downstream of the HEXXGH motif, and its first glutamic acid residue is involved in zinc coordination [65] (Fig. 2A). A water molecule completes the tetrahedral coordination of the zinc ion in eukaryotic DPP3 homologs [59]. In contrast to the eukaryotic enzymes, btDPP3 features two water molecules that are bound to the zinc ion, resulting in a square pyramidal coordination [61].

Bezerra et al. [60] demonstrated that tynorphin binds to DPP3 with high specificity by the formation of a β-strand of its first three residues. This β-strand binds to the five-stranded β-core of the enzyme's lower domain in an antiparallel manner [60]. Moreover, the N-terminus of the bound substrate is anchored tightly to the side chains of Glu-316 and Asn-394 and the carbonyl-group of the main chain of Asn-391 (Fig. 2B).

In the closed confirmation of DPP3, the substrate's N-terminus is completely buried by the enzyme, whereas the binding cleft becomes more spacious and is even connected to the exterior via a tunnel towards the position of the C-terminus of the substrate. This structural feature contributes to DPP3's ability to accommodate peptides of a length between four and ten residues [60]. In addition, a series of conserved arginines, named 'Arg anchors', is found at varying distances from the catalytic zinc ion. This allows for substrates of different lengths to form a salt bridge between their C-termini and the guanidinium group of the properly positioned Arg anchor in respect of the substrate length [59,60]. In yDPP3, for example, Baral et al. [59] identified the side chain of Arg-582 to bind the C-terminus of tetrapeptide substrates and proposed this residue to interact with pentapeptides as well. Similarly, it was suggested that hexa- and hepta-peptides would bind to Arg-674, while longer substrates with up to ten amino acids interact with Arg-671 or Arg-664.

Apart from the full length, canonical hDPP3 isoform (Isoform1, UniProtKB Q9NY33-1), two additional isoforms, termed isoform 2 and isoform 4, have been deduced (Fig. 3). Isoform 2 (UniProtKB Q9NY33-2) lacks 420 of the 737 amino acids compared to isoform 1. The missing section comprises amino acids 182 to 601 including the conserved zinc-binding motifs (HELLGH and EECRAE), the site of interaction to the N-terminus of the substrate and the mechanical hinge region. Thus, it is very unlikely that this isoform has peptidase activity. In contrast, isoform 4 (UniProtKB Q9NY33-4) lacks only the amino acids 91 to 120 from the canonical sequence, and therefore, still features all structural elements that are required for a functional enzyme. However, neither the presence of isoform 2 and 4 nor their respective mRNAs has been confirmed experimentally thus far

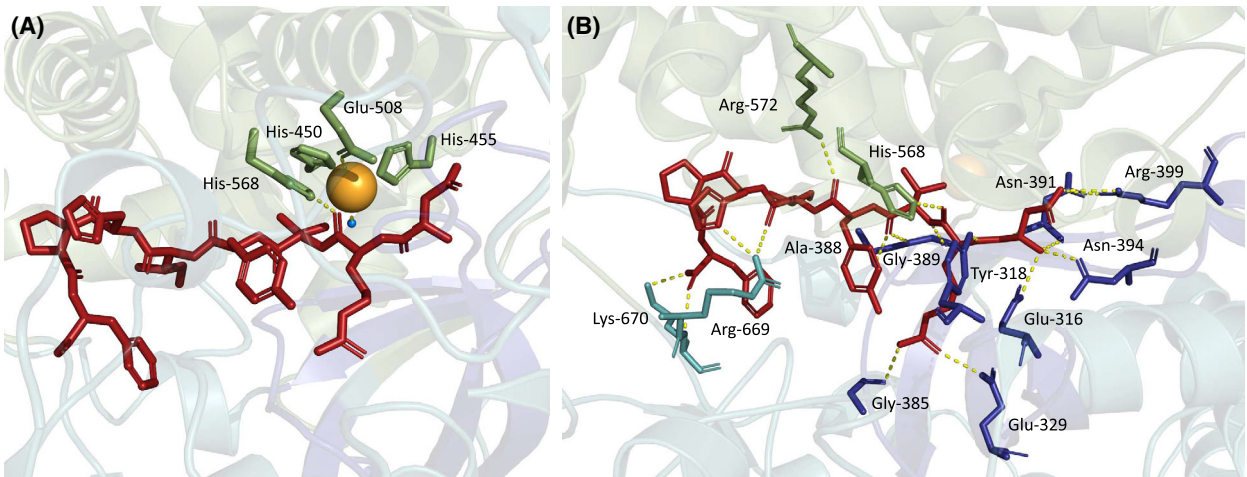


**Fig. 2.** Zinc and substrate coordination of DPP3. (A) Coordination of the zinc ion by residues of the enzyme's conserved motifs. Residues belonging to the enzyme's upper domain are shown in green. The zinc ion is depicted as an orange sphere, the water molecue as a blue sphere and angiotensin II is shown in red. Polar interactions are highlighted as yellow dashed lines. (B) Coordination of angiotensin II by DPP3. The enzyme interacts with the substrate mainly via the lower domain. Residues belonging to the lower domain are shown in blue, with residues forming the five-stranded β-core being highlighted in dark blue. Figures were created in PyMol with the PDB entry 5E2Q, the zinc ion and the water molecule were superposed from PDB entry 5E33.

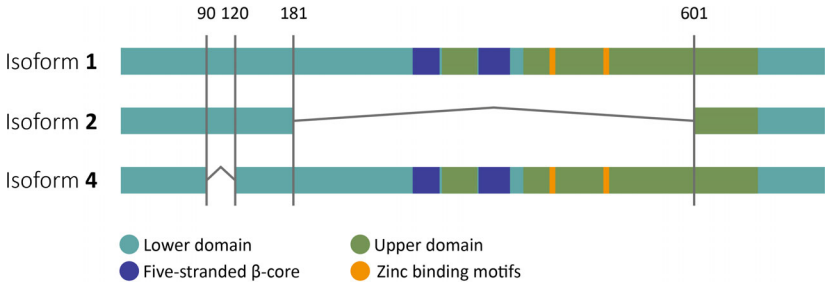


**Fig. 3.** Isoforms of hDPP3. Schematic of the sequence of full length hDPP3 (Isoform1) and its most relevant regions in comparison with the deduced alternative hDPP3 sequences Isoform 2 and 4.

[31]. Moreover, Prajapati et al. [68] demonstrated that isoform 4 is not transcribed in human brain (U87MG), uterus/cervix (SiHa), tongue SCC4 and ovarian (SKOV1) cells. In contrast, two DPP3 isoforms have been verified experimentally from *D. melanogaster* (89 and 82 kDa) [32] and from cockroach (80 and 76 kDa) [33].

Although DPP3 is quite unspecific with respect to the length and sequence of its peptide substrates, it always cleaves off two amino acids from the N-terminus of the substrate. The dominant basis for the enzyme's dipeptidyl-peptidase specificity is the binding of the substrate's N-terminal amino group in proper distance to the catalytic zinc ion [59]. This required proximity is set up by the domain movement, which brings the catalytic machinery located at the upper lobe close to the carbonyl group of the substrate's scissile amide bond [60]. The catalytic mechanism of hDPP3 is proposed to be a water-mediated hydrolysis, akin to other metalloenzymes such as thermolysin [69] and neprilysin [70]. Glu-451 from the HEXXGH motif has been revealed by Bezerra et al. [60] to be properly positioned to act as a general base to deprotonate the zinc-bound water molecule in order to activate it for the nucleophilic attack onto the amide bond. His-568 stabilizes the tetrahedral intermediate by forming a hydrogen bond to the carbonyl group of the substrate's peptide bond that is cleaved by the enzyme [60,62] (Fig. 4A). Notably, Glu-451 and His-568 are invariant residues among the known DPP3s emphasizing their crucial role in catalysis [65]. Interestingly, the Glu-451 corresponding Glu-449 in btDPP3 is not oriented towards either of the two zinc-coordinated water molecules [61], suggesting another mechanism to take place in btDPP3.

In contrast to the water-mediated hydrolysis that was proposed to be responsible for fast substrate cleavage by DPP3, an anhydride mechanism of peptide hydrolysis was suggested to take place at the scission of inhibitory peptides, better characterized as 'slow' substrates (Fig. 4B). 'Slow' substrates such as tynorphin or IVYPW fit into the binding pocket of DPP3 and, thus, block the enzyme's ability to cleave other substrates until the 'slow' substrate is degraded over a period of time. It was discovered that in complex with the 'slow' substrate IVYPW, the zinc-coordinated water molecule is missing. Instead, the peptide is coordinated directly to the zinc ion and the side chain of Glu-451, located only 4 Å from the scissile peptide bond in this complex, was presumed to act as a nucleophile. Thus, 'slow' peptides such as IVYPW or angiotensin III appear to force an energetically less favoured anhydride mechanism of peptide hydrolysis, similar to carboxypeptidase A, by displacing the zinc-bound water molecule [43,59,62,71].

## Substrates and inhibitors

*In vitro* studies showed that DPP3 hydrolyses a broad range of synthetic and physiological substrates with good affinities towards angiotensins and opioid peptides (Table 2). Another widely studied substrate for DPP3 is the synthetic Arg-Arg-β-naphthylamide (Arg-Arg-βNA), which forms the fluorogenic substrate β-naphthylamide upon being hydrolyzed by DPP3. Consequently, it is routinely used to assay DPP3 activity [20,31]. Tynorphin and other synthetic hemorphin-like peptides have been reported to inhibit various enkephalin-degrading enzymes [72]. However, as described earlier, these peptides were found to be hydrolysed by DPP3 over a period of time and are therefore slow substrates rather than inhibitors [62].

Due to the broad substrate specificity, the entire range of peptides accepted by DPP3 is still unknown. From what is known about DPP3's mode of substrate binding, a tight interaction between the enzyme and the substrate's N-terminus is crucial for complex formation. This provides a rationale for the finding that substrates are not recognized by DPP3 after modification of the N-terminus, for example, by acetylation [20]. Moreover, the first three N-terminal amino acid residues of the substrate need to be able to form a β-strand in order to bind to the β-core of the enzyme in an antiparallel fashion [60,62]. In addition, DPP3 preferably cleaves the dipeptides Arg-Arg, Ala-Arg, Asp-Arg or Tyr-Gly from the amino termini of

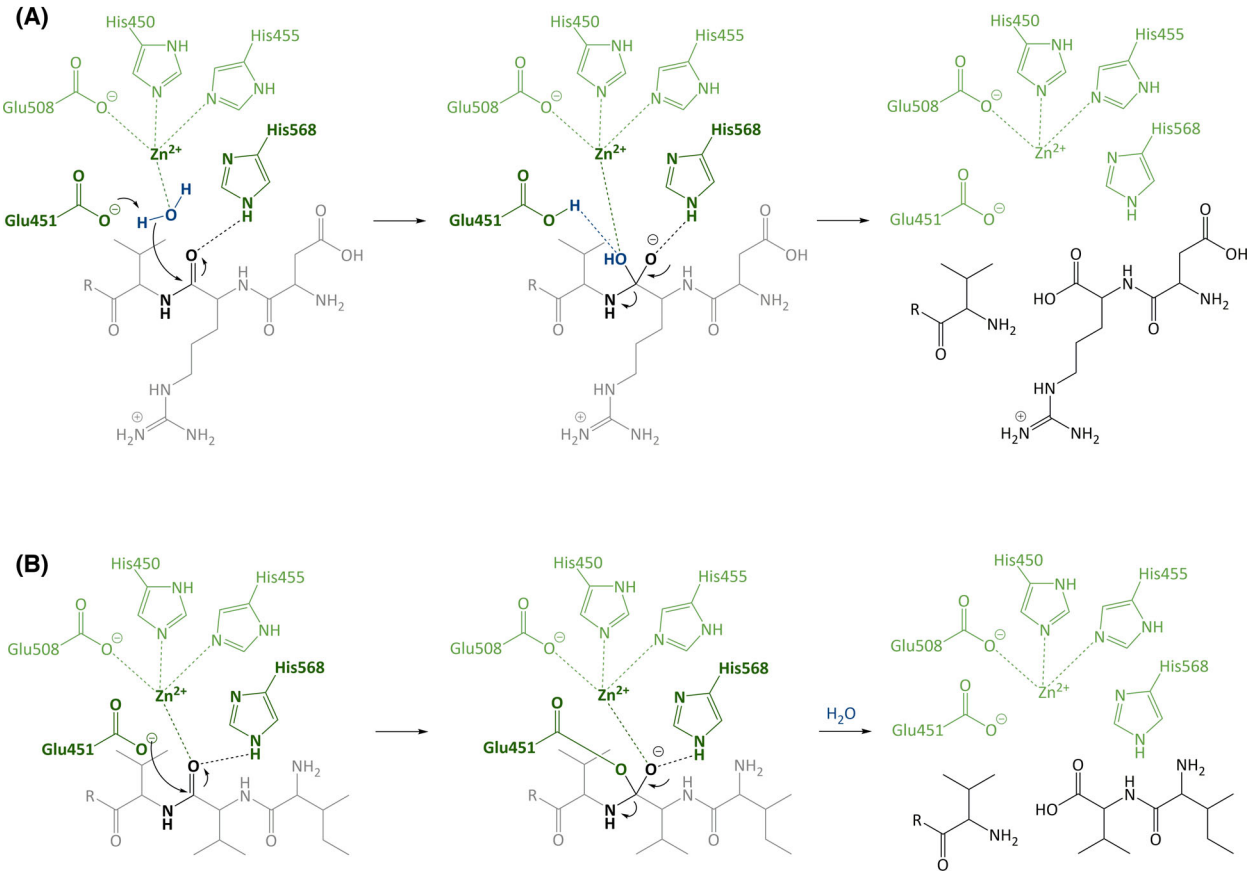


**Fig. 4.** Possible peptide hydrolysis mechanisms catalysed by hDPP3. (A) Water-mediated hydrolysis proposed to take place in the cleavage of 'good' substrates, shown with angiotensin II. Residues of hDPP3 are shown in green, water is depicted in blue and the substrate is shown in black. (B) Anhydride mechanism of peptide hydrolysis that is suggested to occur when a 'slow' substrate is degraded by hDPP3, shown with IVYPW.

oligopeptides. Peptides with the N-terminal dipeptides Ala-Ala, Ser-Tyr, Leu-Ala, Gly-Arg, Gly-Phe, His-Ser or Met-Ser, on the other hand, are not accepted as substrates [40].

Several small non-peptidic molecules have been described as (potent) inhibitors of DPP3. Cysteine peptidase inhibitors such as *p*-chloromercuriphenylsulphate, *p*-hydroxy-mercuribenzoate and *p*-chloromercurisulfonate [20,29,30,36,50,54,63,73–78] have been shown to significantly inhibit mammalian DPP3. Moreover, DPP3 is inhibited by serine peptidase inhibitors such as tosyl-L-phenylalanyl chloromethyl ketone, phenylmethylsulfonyl fluoride and 3,4-dichloroisocoumarin [29,30,36,50,54,63,73–79]. In addition, heavy metal ions like $Cd^{2+}$, $Cu^{2+}$, $Pb^{2+}$ and $Mn^{2+}$, amongst others, were found to exhibit inhibitory properties towards DPP3 [54,73–75]. Due to the enzyme's nature as a metallopeptidase, its susceptibility to metal chelating agents such as EDTA [20,29,30,32,36,50,54,63,73,74,76,77,79] comes as little surprise. However, none of the aforementioned inhibitors are specific for DPP3. Fluostatins A and B, natural products isolated from *Streptomyces* sp. TA-3391, are potent inhibitors of placental DPP3 with only a weak inhibitory activity towards DPP1, DPP2 and DPP4. The IC50 values of fluostatins A and B are 1.44 and 74.0 μM, respectively [80]. Agić et al. [81] investigated diamidino substituted dibenzimidazoles as potential new non-peptide inhibitors. The study revealed that eleven of the twelve tested compounds inhibit human erythrocyte DPP3. The two most promising compounds feature cyclized amidino groups coupled to the benzimidazole moiety as well as additional aromatic residues and reach IC50 values of 1.7 and 2.8 μM, respectively (Table 3).

Moreover, these inhibitors do not affect the activity of the serine peptidase DPP4, indicating their specificity towards DPP3 [81].

Deniau et al. [47] recently described the development of a humanized monoclonal antibody with IgG1 backbone, named Procizumab (PCZ) that is directed against $hDPP3_{474\text{-}493}$. This sequence represents an

**Table 2.** Important physiological and synthetic DPP3 substrates.

| Substrate | Sequence | $K_m$ [μM] | $K_i$ [μM] | $k_{cat}$ [$s^{-1}$] | References |
|---|---|---|---|---|---|
| Angiotensins | | | | | |
| Angiotensin II | Asp-Arg-Val-Tyr-Ile-His-Pro-Phe | 8.40 ± 0.04 | N/A | 0.25 ± 0.007 | [43] |
| | | 3.7 | | 0.56 | [44] |
| Angiotensin III | Arg-Val-Tyr-Ile-His-Pro-Phe | N/A | 0.021 ± 0.003 | N/A | [123] |
| Angiotensin IV | Val-Tyr-Ile-His-Pro-Phe | 1.7 | N/A | 4.7 | [44] |
| Angiotensin (1-7) | Asp-Arg-Val-Tyr-Ile-His-Pro | 1.95 ± 0.20 | N/A | 0.14 ± 0.003 | [43] |
| Angiotensin (1-5) | Asp-Arg-Val-Tyr-Ile | 12.50 ± 2.20 | N/A | 0.35 ± 0.002 | [43] |
| Enkephalins | | | | | |
| Leu-enkephalin | Tyr-Gly-Gly-Phe-Leu | 6.5 | 3.65 ± 0.60 | 9.0 | [42] |
| Met-enkephalin | Tyr-Gly-Gly-Phe-Met | N/A | N/A | N/A | [60] |
| Endorphins | | | | | |
| Endomorphin-1 | Tyr-Pro-Trp-Phe-NH2 | 8.1 ± 3.7 | 5.00 ± 0.12 | 5.0 ± 0.9 | [42] |
| Endomorphin-2 | Tyr-Pro-Phe-Phe-NH2 | N/A | 2.49 ± 0.64 | N/A | [42] |
| Human β-casomorphin | Tyr-Pro-Phe-Val-Glu-Pro-Ile | N/A | 0.56 ± 0.28 | N/A | [42] |
| Proctolin | Arg-Tyr-Leu-Pro-Thr | 4.0 | N/A | N/A | [33] |
| Other peptide substrates | | | | | |
| | Leu-Arg-Arg-Ala-Ser-Leu-Gly | N/A | N/A | N/A | [124] |
| | Leu-Trp-Met-Arg-Phe-Ala | N/A | N/A | N/A | [124] |
| | Val-Leu-Ser-Glu-Gly | N/A | N/A | N/A | [124] |
| | Tyr-Gly-Gly-Phe-Leu | N/A | 115.0 | N/A | [29] |
| | Asp-Tyr-Met-Gly-Trp-Met | N/A | 35.7 | N/A | [29] |
| | Arg-Arg-Lys-Ala-Ser-Gly-Pro | N/A | N/A | N/A | [29] |
| Dipeptidyl derivatives | | | | | |
| | Arg-Arg- βNA | 3.7 ± 1.1 | N/A | 21.5 ± 2.1 | [123] |
| | Arg-Arg-MNA | N/A | N/A | N/A | [50] |
| | Arg-Arg-NH-Mec | 55 | N/A | N/A | [29] |
| | Arg-Arg-MCA | N/A | N/A | N/A | [28] |
| | Ala-Arg-NHNap | N/A | N/A | N/A | [124] |
| | Arg-Arg-NHNap | N/A | N/A | N/A | [124] |
| | Leu-Arg-NHNap | N/A | N/A | N/A | [124] |
| Synthetic spinorphin derivatives | | | | | |
| Spinorphin | Leu-Val-Val-Tyr-Pro-Trp-Thr | N/A | 2.42 ± 0.98 | N/A | [72] |
| | | | 7.82 ± 0.91 | | [40] |
| Tynorphin | Val-Val-Tyr-Pro-Trp | N/A | 0.075 ± 0.012 | N/A | [72] |
| | | | 2.67 ± 0.58 | | [40] |
| | Leu-Val-Tyr-Pro-Trp | N/A | 0.780 ± 0.105 | N/A | [40] |
| | Tyr-Val-Tyr-Pro-Trp | N/A | 0.339 ± 0.022 | N/A | [40] |
| | Phe-Val-Tyr-Pro-Trp | N/A | 0.277 ± 0.065 | N/A | [40] |
| | Trp-Val-Tyr-Pro-Trp | N/A | 0.126 ± 0.015 | N/A | [40] |
| | Ile-Val-Tyr-Pro-Trp | N/A | 0.100 ± 0.011 | N/A | [40] |

unstructured, surface exposed loop region close to the active site of DPP3 that is conserved in all mammals. PCZ was found to efficiently inhibit hDPP3 with a maximum inhibition of 73.3%, presumably due to the steric blockage of DPP3's domain motion. Furthermore, DPP3 inhibition by PCZ was demonstrated *in vivo* in mouse and rat models [47,58]. The latest approach to generate specific DPP3 inhibitors is the design of pseudopeptides. Ivokovic et al. [82] described the synthesis of two epimers that feature the main tynorphin scaffold, where the scissile peptide bond is replaced by non-cleavable hydroxyethylene isostere. Both, the (*S*)- and the (*R*)-epimer (named SHE and HER, respectively) inhibit hDPP3 *in vitro* (Table 3) and HER was additionally shown not to be degraded by the enzyme over a period of 24 h [82].

## Involvement in physiological functions

### DPP3 as a modulator of the renin-angiotensin system

The renin-angiotensin system (RAS) plays a crucial role in the regulation of several physiological parameters, such as blood pressure and electrolyte

**Table 3.** Specific DPP3 inhibitors.

| Inhibitor | *Km* [μM] | $K_i$ [μM] | $IC_{50}$ [μM] | References |
|---|---|---|---|---|
| Fluostatins | | | | |
| Fluostatin A | 10.5 | 14.2 | 1.44 | [80] |
| Fluostatin B | N/A | N/A | 74.0 | [80] |
| Amidino benzimidazole compounds | | | | |
| 2,2′-((2,4-diphenylcyclobutane-1,3-diyl)bis(1H-benzo[d]imidazole-2,6-diyl))bis(4,5-dihydro-1H-imidazol-3-ium) | 12.00 ± 1.58 | 0.20 ± 0.007 | 2.8 | [81] |
| 2,2′-((2,4-bis(2-chlorophenyl)cyclobutane-1,3-diyl)bis(1H-benzo[d]imidazole-2,6-diyl))bis(4,5-dihydro-1H-imidazol-3-ium) | N/A | N/A | 1.7 | [81] |
| Antibody | | | | |
| Procizumab | 0.0096 ($K_d$) | N/A | 0.048 | [47] |
| Hydroxyethylene transition state mimetics of tynorphin | | | | |
| SHE | 23 ($K_d$) | N/A | 98.5 | [82] |
| HER | 11 ($K_d$) | N/A | 13.8 | [82] |

homeostasis. The coordinated effects on the heart, kidney and blood vessels are mediated by a set of peptides that are generated from angiotensinogen: first, renin releases angiotensin I (ANG I), a decapeptide from the precursor protein (> 350 amino acids), and second, angiotensin-converting enzyme removes a dipeptide from the C-terminus to generate angiotensin II (ANG II). ANG II is the primary effector peptide of the RAS—affecting functions of almost all organs, including heart, kidney and vasculature—with both physiological and pathophysiological implications [83]. Recently, it has been shown that DPP3 is also present in low levels (median value of 15 $ng{\cdot}mL^{-1}$) in the circulation of healthy individuals [45,55]. The fact that DPP3, along with its best described substrates angiotensins, is co-located in the circulatory system laid the foundation for the hypothesis that DPP3 may affect the RAS and potentially impact on hemodynamics and the physiology of the cardiovascular system.

However, the mechanism governing the release of primarily intracellular DPP3 into the extracellular space is still unclear. Wilson et al. [84] recently demonstrated DPP3 activity in the culture media of human HK-2 cells, indicating a possible secretion or release of intracellular DPP3 [53,84]. Furthermore, Wattieux et al. [85] reported increased DPP3 activity in the culture media after anti-Fas receptor (CD95) antibody-mediated cell death. This leakage was attributed to the disruption of plasma membranes, a process known as secondary necrosis [85]. Apoptotic and necrotic cell death occurring in Fas receptor-mediated death pathways have been described previously [86–89]. Since cell death plays a major role in critical pathological situations, it could be envisioned that intracellular DPP3 might enter the bloodstream due to massive cell death. In a recent article, van Lier et al. [56] similarly suggested a link between progressive cell death and the uncontrolled release of DPP3 into the circulation during shock of various etiologies [56]. In addition, Gombert and colleagues suggested that rapidly elevated post-operative cDPP3 levels are most likely a consequence of massive cell death during invasive surgery [90]. Although this mechanism could explain particularly high DPP3 concentrations in the blood of severely ill patients, the means by which DPP3 reaches the circulation in healthy individuals is not fully clarified. Therefore, further research is needed to provide solid evidence for the processes that lead to the release of DPP3 into the circulation.

ANG II exerts its most important effects through the ANG II receptor type 1 ($AT_1R$) resulting in mostly beneficial outcomes, such as vasoconstriction, $Na^+$/water homeostasis, the activation of the sympathetic nervous system as well as positive inotropic and chronotropic effects on the myocardium. Consequently, ANG II helps maintain blood pressure and the perfusion of vital organs [83,91,92]. The negative effects of ANG II come from its longer exposure effects (which may be pathological). These include proliferation, cardiac hypertrophy and remodeling, hypertrophy and hyperplasia of vascular smooth muscle cells (VSCM) and inflammation [83,91,93]. The distinction between these beneficial short-term effects and the pathological longer exposure effects is the basis for the clinical applications targeting the RAS in critical care described later.

The hydrolytic products of ANG II can have similar (ANG III (2-8) [92]) or opposite effects (ANG (1-7) and ANG IV (3-8) [93]. ANG (1-7) is thought to balance the RAS through activation of an alternative RAS pathway, which opposes the activity of ANG II [93].

Almost all of the above mentioned angiotensins are substrates of DPP3, which puts DPP3 in a role of altering their distribution and availability. Recently, Jha et al. [43] used a DPP3-knockout mouse model to investigate the effect of DPP3 on the levels of angiotensin peptides in the serum ("RAS-Fingerprint"). This study revealed that DPP3 deficiency results in elevated levels of ANG II, III, IV and ANG (1-5) causing increased water intake and formation of reactive oxygen species in the kidneys [43]. Interestingly, these effects were much more pronounced in male knock-out mice indicating that the endocrine system modulates the consequences of DPP3 deficiency. The RAS is disordered in patients with catecholamine-resistant vasodilatory shock [94]. This disturbance was initially attributed to decreased ACE activity and AT1R blockade. However, recent reports show that high DPP3 concentrations are found in the blood of critically ill patients, especially the ones suffering from cardiogenic and septic shock [55,95]. The current evidence suggests that enhanced ANG II degradation by DPP3 is an additional explanation for the RAS disturbances observed in shock patients. These findings point out that, at a biological level, the journey to understand the complexity of the non-classical RAS pathways has just begun. At a clinical level, it suggests that improvement in shock patient management strategies will need to evolve towards the identification of the pathways leading to RAS disturbances and treatment regimes involving RAS-modulating agents to improve patient outcomes.

## DPP3 and oxidative stress

The influence of DPP3 on the Keap1-Nrf2/ARE signal pathway suggest a direct involvement of DPP3 in the oxidative stress response [8,14,31,52,96,97]. It was shown that DPP3 competes with Nrf2 through the ETGE motif to bind to Keap1 and consequently enhances the translocation of Nrf2 to the nucleus, thereby driving the expression of an array of genes encoding anti-oxidative enzymes [96]. More specifically, the binding of DPP3 to Keap1 releases Nrf2, and thus, prevents its degradation through the 26*S* proteasome. As a result, Nrf2 is translocated to the nucleus, where it acts as a transcription factor [96,98–102] (Fig. 5). Thus, DPP3 functions as an activator of the Nrf2-mediated antioxidant response and thereby reinforces the defense mechanism against oxidative stress [10]. Furthermore, it was reported that overproduction of DPP3 in liver cells generated from CBA/H female mice, correlates with high levels of Nrf2 in the nucleus. This increase in Nrf2 was associated with reduced cell damage following hyperoxia-induced oxidative stress [52]. Similarly, it has been shown that the

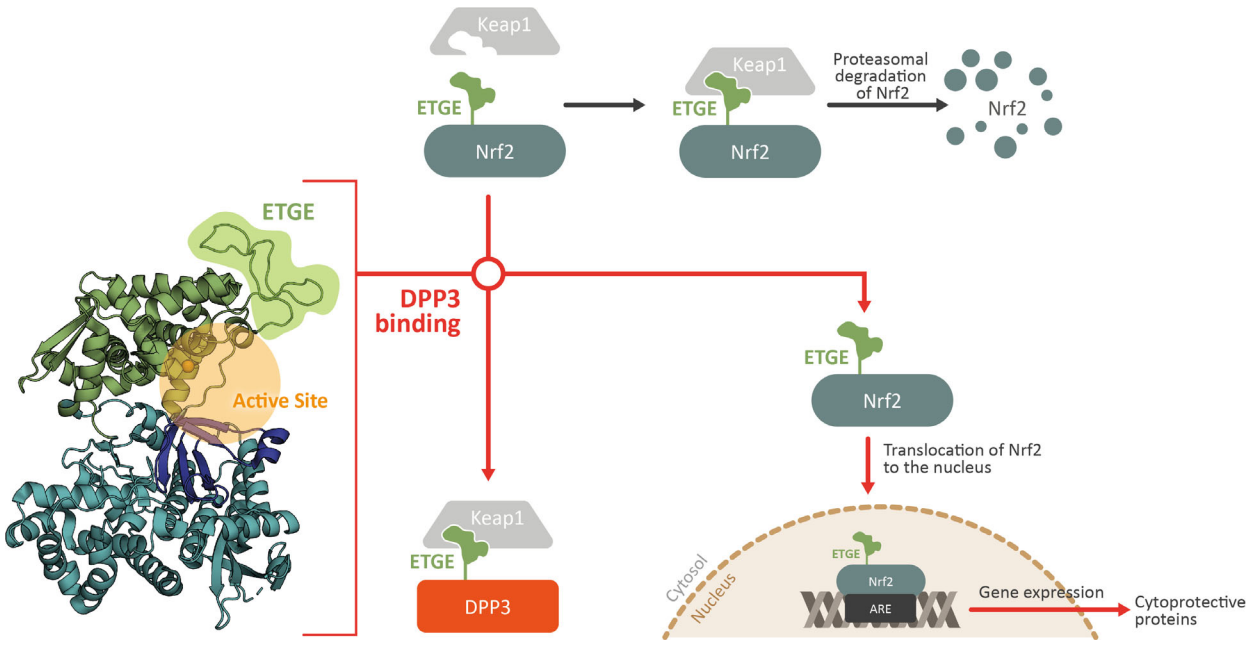


**Fig. 5.** Involvement of DPP3 in the Keap1-Nrf2/ARE signal pathway. Schematic representation of Keap1-Nrf2 signal pathway including the moonlightning activity of DPP3 as an activator of the expression of genes encoding cytoprotective enzymes involved in the oxidative stress response.

Nrf2 mediated oxidative stress response activated by the overproduction of DPP3 can contribute to cancer cell survival [10,38,103,104].

The role of DPP3 in the Keap1-Nrf2/ARE signal pathway was also found to be crucial for bone homeostasis [8]. Menale et al. [8] reported a bone loss phenotype in a *dpp3*-knockout mouse model due to elevated ROS levels and diminished defence against oxidative stress as a consequence of a dysfunctional Nrf2/HO-1 pathway. This impact of DPP3 deficiency on bone development and maintenance was observed only in older animals. In addition, Ren et al. [14] investigated the neuroprotective role of DPP3 in the oxygen-glucose deprivation/reoxygenated (OGD/R) mouse hippocampal neuron cell line HT22, where OGD/R was used to mimic cerebral ischaemia/reperfusion *in vitro*. They showed that inducing cell stress via OGD/R has an effect on DPP3 overproduction in HT22 cells, rationalizing increased ROS production by DPP3 depletion and *vice versa*. A protective role of DPP3 was also demonstrated under conditions, such as apoptosis and inflammation, where the depletion of DPP3 ameliorated caspase-9 activity, and thus, provoked apoptosis of HT22 neurons [14]. This supports the claim that DPP3, among other things, plays an important role in cell survival.

### DPP3 in immune response

Another function of DPP3 that has been reported in several studies is its involvement in inflammation and immune response. DPP3 expression and activity was found in cells of the innate immune system, such as polymorphonuclear granulocytes and monocytes [12,27] and in cells of the adaptive immune system, such as lymphocytes [27]. It was shown that the lack of DPP3 has an impact not only on the overproduction of pro-inflammatory cytokines but also on anti-inflammatory cytokines [8,13,14]. The upregulation of pro-inflammatory cytokines TNFα, IL-1β, IL-6 was also observed in *dpp*3 KO bone marrow cells [8,14] and in macrophages (TNFα) [14]. Those results may indicate a major impact on immunoregulation and the involvement of DPP3 in initiating and maintaining an immunological response. Clearly, this area is currently insufficiently researched and further extensive research is needed.

## Involvement in pathophysiological processes

### Pain modulation

DPP3 was initially described as enkephalinase B owing to its enkephaline-degrading activity [60]. Enkephalins (Leu- and Met- enkephalin), along with endomorphins (endomorphin 1 and 2) constitute part of the endogenous opioid system [12,36] that is involved in pain regulation and modulation [40,41,60]. Interestingly, the catalytic rates ($k_{cat}$) reported for Leu-enkephalin and endomorphin-1 are more than ten times larger than those for the angiotensin peptides suggesting that DPP3 may play a central role in regulating the levels of enkephalins and endomorphins. In fact, Sato et al. [41] reported significantly lower levels of activity of DPP3 in human cerebrospinal fluid in patients with acute pain. Moreover, immunohistochemical staining of rat spinal cord confirmed the co-localization of DPP3 in the superficial laminae of the dorsal horn along with enkephalinergic neurons, indicating its involvement in nociception [40,42,105]. However, in view of the scarcity of information from *in vivo* studies in mammals, the extent to which DPP3 affects nociception is currently unclear.

### DPP3 and cancer

A possible role of DPP3 in cancer development was suggested more than 20 years ago [19] and has received substantial support in recent years [9,10,15–18,103]. Generally, tumorigenesis appears to be associated with higher expression of *Dpp3* resulting in the overproduction of DPP3. This was demonstrated for endometrial and ovarian malignant cancer in comparison to benign and normal tissues [18,19]. More recently, overexpression of *Dpp3* was reported for squamous lung [103], breast [9,10] and colorectal cancer (CRC) [15]. Interestingly, high *Dpp3* expression was also linked to poor prognosis in multiple myeloma, CRC and estrogen positive ($ER^+$) breast cancer [10,15–17]. The strongest evidence for a correlation of *Dpp3* expression and tumorigenesis comes from studies on breast and colorectal cancer [9,10,15,16]. In a novel and integrated computational approach, Li and coworkers [18] analysed 98 whole exome samples from breast cancer patients and scored the sum deleterious effect of all mutations in a gene. This led to the identification of different mutations of *Dpp3* in both estrogen receptor (ER+/−) and progesterone receptor (PR+/−) breast cancer subtypes. They identified a missense mutation leading to the exchange of a glutamate to a lysine residue in position 690 of DPP3 (rs12421620). Accordingly, the authors predict that this amino acid exchange results in a destabilization of a C-terminal α-helix and consequently to a loss of function [16]. The overexpression of *Dpp3* in breast cancer and the associated correlation with poor prognosis was confirmed by Lu et al. [10]. These authors also found that the expression of genes controlled by Nrf2 was significantly increased compared to normal

adjacent tissue suggesting that DPP3 exerts its effect by displacing Nrf2 from the complex with Keap1 leading to its translocation to the nucleus and subsequent gene expression (Fig. 5) [10].

A role of DPP3 in breast cancer was highlighted also by Choy et al. [9]. They analysed the high-throughput transcriptomic profiles of breast cancer samples across multiple datasets in publicly available databases. An Oncomine analysis showed that mRNA levels of *Dpp3* were highly upregulated in breast cancer tissues. Using the Kaplan–Meier Plotter tool and a subsequent multivariate analysis confirmed that high expression levels of *Dpp3* correlated with poor survival of breast cancer patients and were an independent survival determinant. A GeneGO Metacore analysis demonstrated that genes coexpressed with *Dpp3* were involved in cell-cycle related pathways [9]. In the same vein, the study by Tong et al. [15] demonstrated that reduced *Dpp3* expression in colorectal cancer cells significantly inhibited cell proliferation and migration *in vitro*. In addition, the induction of apoptosis and cell cycle arrest was observed. *In vivo*, the downregulation of *Dpp3* expression resulted in a reduced growth rate of tumours. These observation prompted Tong and coworkers to ascribe oncogene-like properties to *Dpp3* [15]. On a molecular level, it was found that DPP3 interacts with cyclin-dependent kinase 1 (CDK1), a central regulatory enzyme of the cell cycle. However, the nature of this interaction and its potential consequences for gene expression and/or biochemical processes in the cell remain to be investigated.

In summary, the current data suggests a role of DPP3 in progression and development of a variety of cancers. However, the molecular mechanisms of its involvement are elusive. On the one hand, mutations in the gene may lead to DPP3 variants with altered biochemical properties that potentially affect its catalytic activity and/or its interaction with Keap1. On the other hand, it is also conceivable that overproduction of DPP3 directly leads to an increased antioxidant response through escape of Nrf2 from the Keap1-complex, and thus, supports cancer cell growth by means of increased protection against ROS. Moreover, the observation that DPP3 interacts with CDK1 may indicate that Keap1 is not the only interaction partner of the protein. Therefore, it is of utmost importance in future research efforts to identify the mechanisms by which DPP3 affects the cellular metabolism of cancer cells. This insight is clearly needed to design strategies to exploit DPP3 as a potential anticancer target.

## The detrimental effect of DPP3 in circulation: Biomarker and drug target?

As noted above, the presence of DPP3 in the vascular system has been established although the mechanisms by which the protein is released from cells have not been addressed adequately. In any case, the low levels of DPP3 found in healthy humans increase dramatically under certain acute conditions such as septic, cardiogenic and vasodilatory shock [45]. Shock is defined as a circulatory failure accompanied by organ hypoperfusion and is associated with various dysregulated molecular mechanisms and high fatality rates of up to 40%. The classification schemes used provide basic guidance for treatment decisions but fail to address the complexity of the molecular pathways leading to the development of these shock syndromes. During shock, the upregulation of ANG II maintains tissue perfusion and is considered an important natural response to the hypotensive state. In accordance with this role, the recent ATHOS-3 trial has revitalized the use of ANG II as an alternative vasopressor in catecholamine-resistant vasodilatory shock (CRVS). However, the substantial increase of DPP3 levels and activity found in shock patients would clearly lead to accelerated degradation of ANG II, and thus, counteract the intravenous administration of ANG II. As a matter of fact, high DPP3 blood levels are associated with higher organ dysfunction scores, the need of cardiovascular support and the development of myocardial dysfunction, refractory shock, acute kidney injury and increased short-term mortality. Not surprisingly, a reduction in DPP3 blood levels following conventional treatment was associated with lower organ support requirements and reduced mortality rates in all of these conditions. Since DPP3 concentrations strongly correlate with DPP3 activity in the blood [45,47] both measurements accurately predict patient illness severity [45–47,57,58]. On the basis of these clinical associations, it is plausible that active DPP3 released in the blood of shock patients is a new factor contributing to the deterioration of vascular tone in shock syndromes by disabling the vasopressor effects of endogenous ANG II.

To further validate the findings of the shock cohort analysis, efficient methods for native DPP3 purification [106] and animal models to study the pathophysiological role of DPP3 were developed. It was shown that (a) DPP3 intravenous injection leads to reduced cardiac and renal function; (b) high DPP3 blood levels induced by acute cardiac stress or septic cardiomyopathy can be inhibited by a humanized monoclonal anti-cDPP3 IgG1 antibody (anti-cDPP3-mAb), which normalized cardiac

function, restored hemodynamic stability and reduced mortality [47]. In summary, DPP3 has emerged as a valuable biomarker to predict the severety of shock syndromes, and importantly, lends itself as an attractive target to attenuate the potentially lethal symptoms. In that context, DPP3 activity could be reduced either by bioceuticals, e.g., antibodies directed against the protein or small molecule inhibitors, as described recently [82].

## Future perspectives

In view of the multitude of physiologic effects reported for DPP3, it is not surprising that the enzyme has received significant attention over the last two decades. The elucidation of the three dimensional structure of DPP3 from various sources has set the stage for mechanistic studies and enables the structure-guided design of highly specific small-molecule inhibitors. Apparently, DPP3 plays distinct roles in intracellular and extracellular (circulatory) physiology, which require carefull dissection in terms of the potential pathologies connected with DPP3 activity. Intracellularly, the role of DPP3 involves the removal of dipeptides from the N-terminus of an as yet unknown array of small peptides as well as the interaction with the Nrf2/Keap1 complex. The latter function is related to the regulation of the cellular antioxidant response with dysfunctions potentially contributing to disease-related processes such as the development and progression of various tumours. The role of DPP3 in cells of the innate and adaptive immune system is yet another exciting area with the potential to improve our understanding of immunologic processes.

In extracellular physiology, the enzymatic activity of DPP3 affects the distribution of angiotensin peptides in the RAS indicating a crucial role as a regulator of this system. This emerging role was highlighted in a series of reports that established circulating DPP3 as a risk factor in the case of acute medical conditions such as heart and kidney failure or septic shock. These recent insights of DPP3 as a predictive biomarker provide a new diagnostic parameter to assess short-term outcomes with the potential to optimize patient stratification, improve prognosis and guide therapy escalation, being a valuable addition to the repertoire of intensive care biomarkers. Equally, this puts DPP3 in the limelight as a target for the development of specific inhibitors holding the promise as an effective intervention strategy in the case of pathologies that correlate with increased DPP3 levels in the cardiovascular system.

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## Conflicts of interest

The authors declare no conflict of interest.

## Author contributions

GM, BH, SJ and PM conceived the review; GM, BH, SS, MS, KS, SJ and PM developed and conceptualized the review; GM, BH, SS, MS, KS and PM wrote the original draft; GM, BH, SS and PM edited and revised the manuscript; GM, BH and PM organized and supervised the final version of the manuscript.

## Data Availability Statement

Data sharing is not applicable to his review article as no new data were created or analysed.

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