


Review

# Thimet Oligopeptidase—A Classical Enzyme with New Function and New Form

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**Abstract:** Peptidases generate bioactive peptides that can regulate cell signaling and mediate intercellular communication. While the processing of peptide precursors is initiated intracellularly, some modifications by peptidases may be conducted extracellularly. Thimet oligopeptidase (TOP) is a peptidase that processes neuroendocrine peptides with roles in mood, metabolism, and immune responses, among other functions. TOP also hydrolyzes angiotensin I to angiotensin 1–7, which may be involved in the pathophysiology of COVID-19 infection. Although TOP is primarily cytosolic, it can also be associated with the cell plasma membrane or secreted to the extracellular space. Recent work indicates that membrane-associated TOP can be released with extracellular vesicles (EVs) to the extracellular space. Here we briefly summarize the enzyme's classical function in extracellular processing of neuroendocrine peptides, as well as its more recently understood role in intracellular processing of various peptides that impact human diseases. Finally, we discuss new findings of EV-associated TOP in the extracellular space.



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**Keywords:** thimet oligopeptidase; extracellular vesicles; peptide; enzyme; metallopeptidase; hormone

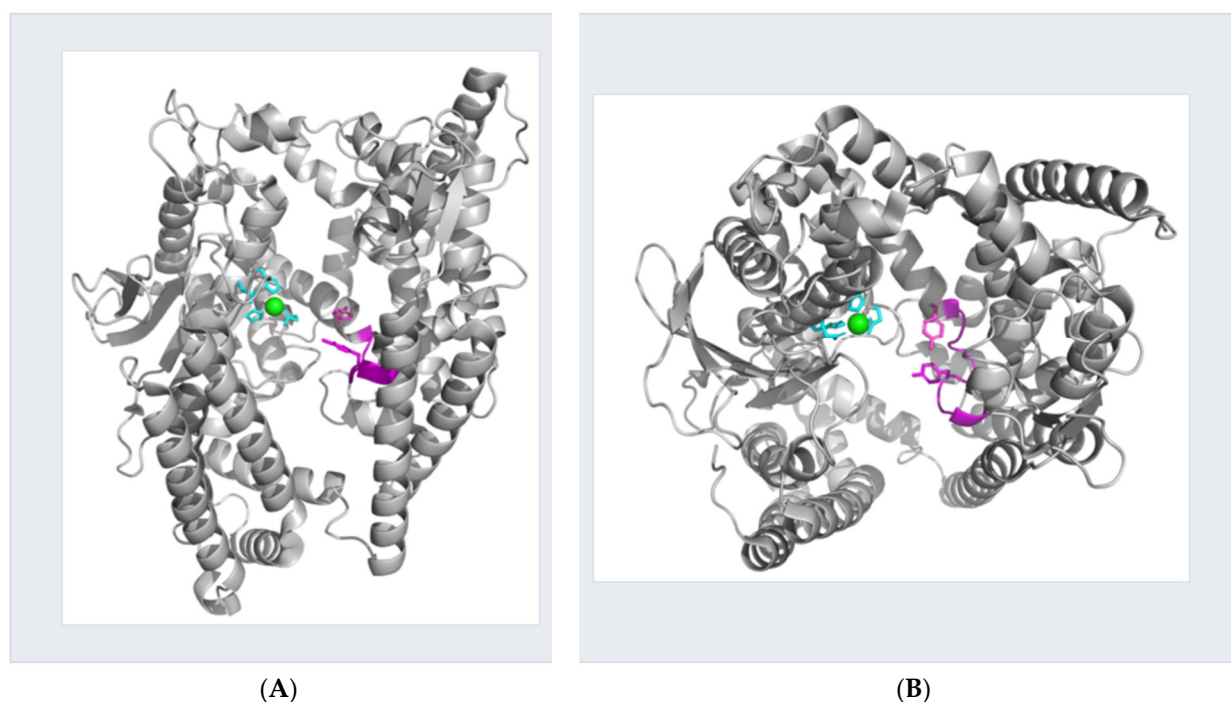
## 1. Introduction: Thimet Oligopeptidase—Classical Function, Regulation, and Structure

Thimet oligopeptidase (TOP), also called endopeptidase 24.15 (E.C. 3.4.24.15), is a zinc metalloendopeptidase of molecular weight 77 kD [1,2]. TOP is found in humans and other animals, and the enzyme hydrolyzes a number of important neuroendocrine and cardiovascular peptides that help regulate physiological and pathological conditions [2–4]. In 1980, Horsthemke and Bauer first characterized TOP as a neutral endopeptidase which degrades gonadotropin-releasing hormone (GnRH, also called LHRH) [5]. This enzyme was primarily detected from soluble extracts of bovine anterior pituitary tissue and was shown to be effective at cleaving GnRH [5]. Later, Orłowski et al. purified the enzyme from rat brain [6] and rat testes [7] and investigated the determination of specificity with synthetic and natural peptides of this soluble metalloendopeptidase [6]. Given the enzyme activity of TOP and its ability to degrade several bioactive peptides, Orłowski et al. proposed a potential function of the metalloendopeptidase in neuropeptide metabolism [6]. The study later found that TOP can cleave and process not only GnRH, but also bradykinin and neurotensin [7].

TOP is regulated at the level of transcription by both positively and negatively acting elements in a cell-specific manner [8,9]. For example, we have shown that expression of TOP is downregulated by estradiol in steroid-sensitive regions of the brain [10,11]. However, there are a number of other potential mechanisms for regulation of TOP levels, activity, and distribution. TOP is dependent on redox state, with activity greatly reduced by dimerization or higher-order oligomerization via intermolecular disulfide linkage of surface cysteine residues [12–14]. Although activation of the enzyme by thiol reagents was

originally thought to occur only in vitro, more recent studies suggest that S-glutathiolation occurs in vivo [15,16], and that modulation of TOP activity may occur via a redox cascade [17]. Phosphorylation is another post-translational modification that affects TOP activity and possibly function. When TOP is phosphorylated in vitro, its affinity and turnover of GnRH is altered [18], and autophosphorylation following exposure to ATP also affects TOP activity [19]. In addition, compartmentalization and secretion of TOP can be modulated by a variety of factors, including calcium [20] and 14-3-3 epsilon [21]. Other non-conventional modulators of TOP activity have been reported, such as sleep deprivation [22] and mechanical shear [23,24], possibly mediated by G proteins.

The catalytic unit of TOP contains a HEXXH zinc-binding motif in which the two histidine residues serve as ligands to a catalytic  $Zn^{2+}$  ion (Figure 1). Site-directed mutagenesis of the rat enzyme established that His473, Glu474, and His477 make up the HEXXH motif, with Glu502 serving as the third zinc ligand [25]. Based on this active site architecture, TOP is categorized as a gluzincin, in which class thermolysin is the prototypical member [26,27]. Extensive mechanistic studies on thermolysin and related enzymes have shown that glutamate within the HEXXH motif serves as a general acid-base catalyst [26,28,29], and this is also the case for TOP [25]. Enzymes within the group are known to exhibit a bell-shaped pH profile for activity with an optimal pH around 7 [30,31]. A similar bell-shaped profile for the activity of TOP provided additional mechanistic support for its placement within this active site architecture [32].



**Figure 1.** Crystal structure of thimet oligopeptidase. Ribbon representation of open structure of human thimet oligopeptidase (PDB 1s4b). Side chains of the residues forming the catalytic unit are highlighted in cyan with the metal-ion depicted as a green sphere. The flexible loop, including the side chains of Tyr-605 and Tyr-612, are shown in magenta. (A) View perpendicular to the peptide-binding crevice. (B) View parallel to the peptide-binding crevice. Images were created using PyMOL software (Version 2.5.0).

TOP belongs to the M3 family of peptidases, which includes homologs distributed across organisms from bacteria and fungi to mammals [1]. X-ray structures for 5 members of this family, including TOP, demonstrate that all share a bi-lobal, primarily alpha-helical structure with a 40 Å deep crevice separating the lobes, at the bottom of which resides the active site zinc [3,33–36]. Depending on the conditions of crystallization, the enzymes appear to have an open [3,33,36] or closed conformation [34,35,37]. While TOP has yet

to be crystallized in a closed conformation, several lines of evidence, including those from our laboratory, point to the role of an induced fit as a means for substrate selectivity controlled by repositioning of conserved, catalytic Tyr residues: Tyr residues 605 and 612 in domain II and opposite the enzyme active site have been shown to be critical for efficient catalysis [32,38]. In the open TOP structure these residues are too distant from the active site to interact with a bound substrate. Further, a flexible loop (residues 599–611) is proposed to play a role in proper positioning of the Tyr residues when TOP binds its diverse array of peptide substrates [39]. In addition, substrate specificity is altered by partial denaturation of the enzyme [40]. Recently, the structure of the related enzyme human neurolysin (NLN) was solved in a closed conformation [37]. Comparison to the open form of rat NLN revealed that the secondary structure and fold of the domains were unchanged with structural changes confined to short loops connecting the domains at the bottom of the catalytic cleft. The largest structural change occurring in the loop 600–612 contained conserved Tyr residues.

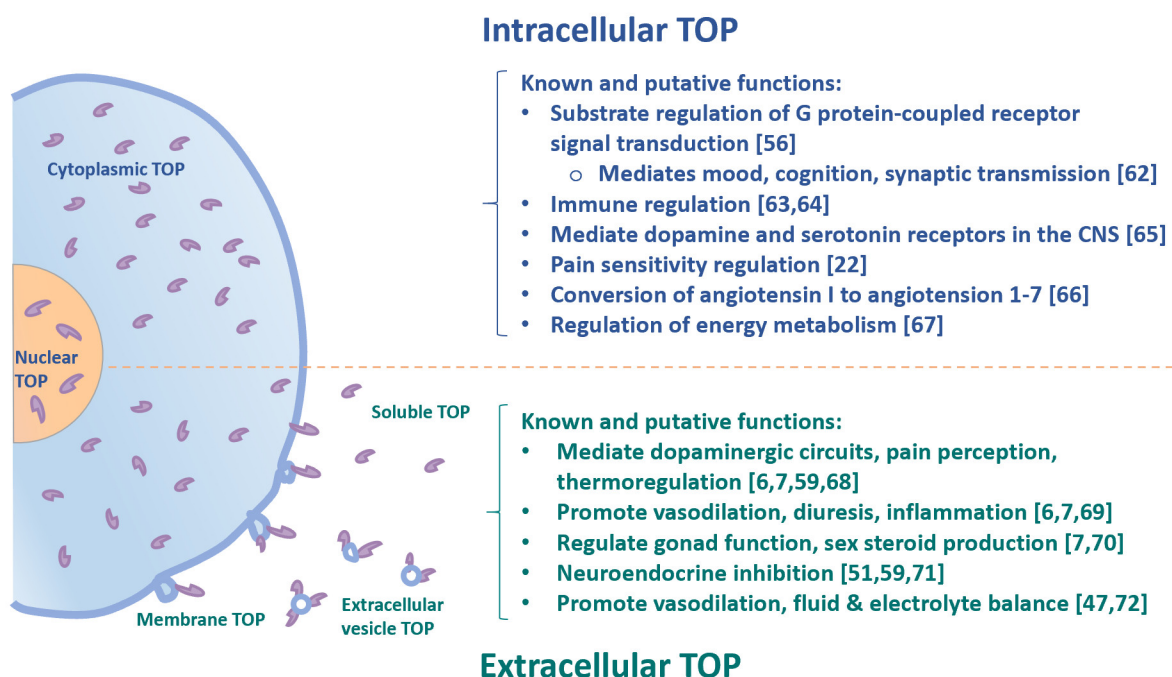
## 2. TOP Plays Dual Roles as an Intracellular and Extracellular Peptidase

TOP is expressed in a wide range of mammalian tissues and cell types, with the highest levels of TOP found in the brain, pituitary gland and testis, in keeping with its neuroendocrine function, while lower levels of TOP can be detected in liver, kidney, lung and spleen [2,4,41–48]. The enzyme is primarily cytoplasmic, suggesting its participation in general intracellular degradative processes [2], while there is also significant presence in the nucleus [49,50]. Altogether, intracellular TOP accounts for 75% of the total enzyme [2]. Given its prevalence in the nervous system and its ability to cleave bioactive peptides *in vitro*, TOP has been hypothesized to play an important role in the metabolism of neuropeptides in the extracellular space [2], i.e., for processing GnRH, bradykinin, and neurotensin in blood (Figure 2). However, it is not clear how TOP reaches the extracellular space. As we discuss in a later section, emerging evidence suggests TOP may be secreted to the extracellular space as a soluble protein or released from the cell on extracellular vesicles.

Traditionally, TOP has been classified as a metabolizing enzyme which terminates neuropeptide function through hydrolysis, such that these substrates are unable to bind to their corresponding receptors [51,52]. However, other studies also demonstrate that TOP plays several alternative roles in the physiological metabolism of various neuropeptides [53]. First, it has been reported that TOP can transform inert precursor neuropeptides into their physiologically bioactive forms [41]. Second, the enzyme can convert one peptide into another bioactive peptide, which can either bind to a new receptor or initiate various downstream signaling pathways upon binding to the initial receptor [54]. Additionally, studies have shown that TOP can work as a bio-modulatory enzyme, whereby a peptide product will act in opposition to its original parent peptide [55]. Given its many roles in the metabolism of neuropeptides, TOP may also play an important role in cell regulation. In fact, studies with rat brain tissue have shown that TOP and its relevant peptide substrates are regulators of the G protein-coupled receptor (GPCR)-mediated signal transduction pathways (Figure 2) [56].

As noted, bioactive peptide signals serve as important chemical messengers for the mediation of intercellular communication in multicellular organisms by binding to cell surface receptors of target cells. Since most peptides are initially synthesized as inactive precursors that require further processing, hydrolysis by peptidase enzymes is required to gain full functionality [2,4]. However, peptide processing is not uniquely intracellular, and may also continue in the extracellular milieu. Thus, extracellular peptidases such as TOP play a crucial role in the physiological generation and regulation of bioactive peptide signals. In the extracellular space, TOP may either convert an inert precursor to an active moiety, or proteolyze peptides to generate products unable to activate receptors. TOP is known to hydrolyze only short peptides of fewer than 20 residues in length, and to recognize greatly variable cleavage sequences depending on the specific substrates [51,57–59].

Established physiological substrates of TOP have been shown to include a number of important bioactive peptides of the nervous system, reproductive axis, pain nociception, as well as the cardiovascular and renal systems, such as GnRH, neurotensin, somatostatin, opioids, bradykinin, and angiotensin [6,7,42,47,51,55,59–61]. TOP can inactivate vasodilatory peptide bradykinin [2], and thus may be involved in cardiovascular homeostasis (Figure 2).



**Figure 2.** Schematic overview of intracellular and extracellular TOP and its biological functions.

### 3. Cytosolic TOP Can Process Intracellular Peptides and Serve as a Natural Regulator of Intracellular Signaling

Although the majority of published works have focused on TOP function in the cleavage of a number of bioactive peptides in the extracellular space [6,7,42,47,51,55,59–61], its role in processing intracellular bioactive peptides has increasingly been investigated over the past decade [56]. Since TOP is primarily a cytoplasmic enzyme, it is logical that it would act as a natural regulator of cell signaling through processing of intracellular peptides (Figure 2) [56]. Berti et al. analyzed the intracellular peptides in human embryonic kidney 293 cells with a quantitative peptidomimetic approach and confirmed that TOP can cleave intracellular substrates that may be used for antigen presentation [62]. Cunha et al. found that TOP is physiologically relevant in the cleavage of intracellular peptides that help to control signal transduction of G protein-coupled receptor (GPCR) [56]. Significant changes in the intracellular peptide composition may disturb cell homeostasis in certain pathological conditions, as GPCRs constitute many cell surface receptors that mediate certain key processes, including mood, cognition, pain, appetite, and synaptic transmission [63]. In order to study the effects of TOP in vivo, Dos Santos et al. generated TOP knockout mice (KO) [64]. The TOP KO mice showed different intracellular peptide ratios, with altered mRNA expression of serotonin 5HT<sub>2a</sub> receptor, proteasome beta5, and dopamine D<sub>2</sub> receptor, as compared to WT mice. In addition, TOP KO mice exhibit depressive-like behavior, as well as attention and memory retention deficits. This suggests the role of TOP on intracellular peptide metabolism and its consequent effects on receptors of serotonin and dopamine, which are known mediators of neuronal communication in many psychiatric disorders [64]. In addition, Visniauskas et al. reported that sleep deprivation decreases TOP activity in the striatum and hypothalamus, which can potentially alter the processing of neuropeptides, and that processing of opioid peptides by TOP may be related to the increase in pain sensitivity in rats [22].

TOP has also been shown to have a protective role in Alzheimer's disease (AD) [65–67]. Amyloid- $\beta$  ( $A\beta$ ) peptide deposition in the brain plays a major role in AD pathology, and intracellular  $A\beta$  accumulation is an early-stage event in the disease; it may be a source for extracellular deposits during disease progression [68]. TOP is involved in the processing of amyloid precursor protein (APP) at a beta-secretase cleavage site and in the degradation of  $A\beta$  peptide, a major player in AD pathology [69,70]. Of note, the findings of Koike et al. were in contrast to earlier studies, which had excluded TOP as a candidate beta-secretase [71]. Furthermore, TOP has been found in neurofibrillary tangles and senile plaques in AD autopsic tissue [72]. Pollio et al. demonstrated that overexpression of TOP was neuroprotective against  $A\beta$ -mediated toxicity in primary cortical neurons [67]. Conversely, knockdown of TOP by RNA interference (RNAi) made neurons more vulnerable to  $A\beta$  toxicity [67]. Notably, the authors found an age-dependent increase in TOP expression in the brain tissue of the TgCRND8 transgenic mouse model of amyloid plaque deposition, where TOP co-localized with  $A\beta$  plaque [67]. TOP expression was significantly increased in AD brain tissue compared to non-AD controls in humans [67]. Taken together, these findings suggest that increased expression of neuronal TOP may play a protective and compensatory defense role against increased  $A\beta$  load seen in early stages of AD pathology [67].

Berti et al. have identified the presence of specific intracellular peptides, which cannot be degraded by TOP [62] in the adipose tissue of Wistar rats [73]. They found that these intracellular peptides may be important in insulin-stimulated glucose uptake in 3T3-L1 adipocytes [73], suggesting the physiological function of intracellular peptides and their potential in metabolic disorders, i.e., obesity and insulin resistance. Very recently, the same group further investigated the involvement of TOP in energy metabolism using TOP KO mice [74]. These animals gained 75% less body weight than controls and did not show non-alcoholic fatty liver steatosis or insulin resistance when compared to their wildtype control mice, after 24 weeks of high fat diet. This work has identified the involvement of TOP in energy metabolism regulation, and TOP-regulated intracellular peptides may be responsible for the regulation of energy metabolism and obesity by an unknown mechanism of action [74].

In antigen-presenting cells, cytosolic peptides/proteins destined for degradation are processed by the proteasome. These peptides will then be transported to the cell surface for antigen presentation. Cytotoxic T lymphocytes (CTLs) recognize peptides that are presented by HLA class I molecules on the cell surface. Kessler et al. reported that cytoplasmic TOP can complement proteasome activity to cleave CLE epitopes [75], resulting in HLA class I binding peptide fragments not compatible for antigenic presentation. Thus, intracellular TOP can affect antigen processing and presentation by its endoproteolytic activity. The relatively new function of intracellular TOP in processing cytosolic peptides provides a new appreciation of this classical enzyme for its role in intracellular peptide regulation of mood, cognition, pain, and psychiatric and neurodegenerative diseases, metabolic diseases, and immunological regulatory functions.

#### **4. Effects of TOP in Intracellular Processing of Angiotensin 1–7 and Its Potential Involvement in COVID-19 Pathogenesis**

TOP has long been known to convert angiotensin I *in vitro* to the biologically active peptide angiotensin 1–7 [76]. More recently, studies have found that TOP is involved in the intracellular conversion of angiotensin I to angiotensin 1–7, especially within the mitochondria and the nucleus [77], therefore contributing to the elevated levels of angiotensin 1–7 in tissues and in the circulation. In addition to TOP, other endopeptidases are also involved in the production of angiotensin 1–7 from angiotensin I [78]. Angiotensin-converting enzyme (ACE)/angiotensin II is a classical signaling pathway in which ACE/ACE2 are carboxypeptidases that can correspondingly conduct catalytic processing of angiotensin I/II to produce angiotensin 1–7 [78]. In addition, other carboxypeptidases that convert angiotensin II to angiotensin 1–7 are carboxypeptidase A and prolylcarboxypeptidase [79–81]. All the above

endopeptidases and carboxypeptidases may contribute to the processing of angiotensin II to angiotensin 1–7.

Recent studies have found a potential link between angiotensin 1–7 and COVID-19 pathophysiology [82,83]. Patients with severe COVID-19 illness had significantly increased circulating levels of angiotensin 1–7 and decreased angiotensin II [82,83]. The angiotensin 1–7/angiotensin II ratio was 3-fold higher in COVID-19 patients as compared to that in healthy controls, thus suggesting an increased activity of ACE2 or other angiotensin 1–7 forming activity after COVID-19 infection [82]. Although TOP is an important angiotensin 1–7 forming factor, expression or activity of TOP in COVID-19 patients have not been analyzed. Additionally, TOP has been shown to process angiotensin I to form angiotensin 1–7 in the hippocampus of the brain [84,85], the kidneys [86–89], and the cardiovascular system [90–92]. These organs have all been shown to be vulnerable to direct infection by SARS-CoV-2 virus, further implicating the potential role of TOP in COVID-19 pathophysiology [93–99].

Prevailing evidence highlights the role of ACE2 as the receptor for both SARS-CoV-1 and SARS-CoV-2 (virus of COVID-19 infection) binding and entry into host cells [100,101]. ACE2 is especially abundant in alveolar epithelial cells and vascular endothelial cells of the lung [78]. In fact, the lethality of infection has been associated with the loss of key regulatory factors in the lung related to viral binding suppression of ACE2 [102]. Tan et al. reported regulatory effects of angiotensin 1–7 in ACE2 expression and found organ-specific down regulation of local ACE2 expression by continuous infusion of angiotensin 1–7 in vivo in rats [103]. However, elevated ACE2 expression has been observed in patients with COVID-19 infection [83,104]. One may, thus, expect that TOP may possibly affect ACE2 expression through processing intracellular angiotensin 1–7, therefore affecting vulnerability of the cells of given tissue/organs to SARS-CoV-2 infection [82,83]. Interestingly, Soria-Castro et al. [105] recently reported that COVID-19 patients showed increased carboxypeptidase A3 (CPA3), which is also known for its function in converting angiotensin II to angiotensin 1–7 [81]. Another enzyme prolylcarboxypeptidase that can catalytically convert angiotensin II to angiotensin 1–7 may also be involved in COVID-19 pathophysiology [106]. Taken together, these findings suggest potential involvement of TOP or other endopeptidases and carboxypeptidases in the pathophysiology of COVID-19 infection is an area worthy of further investigation.

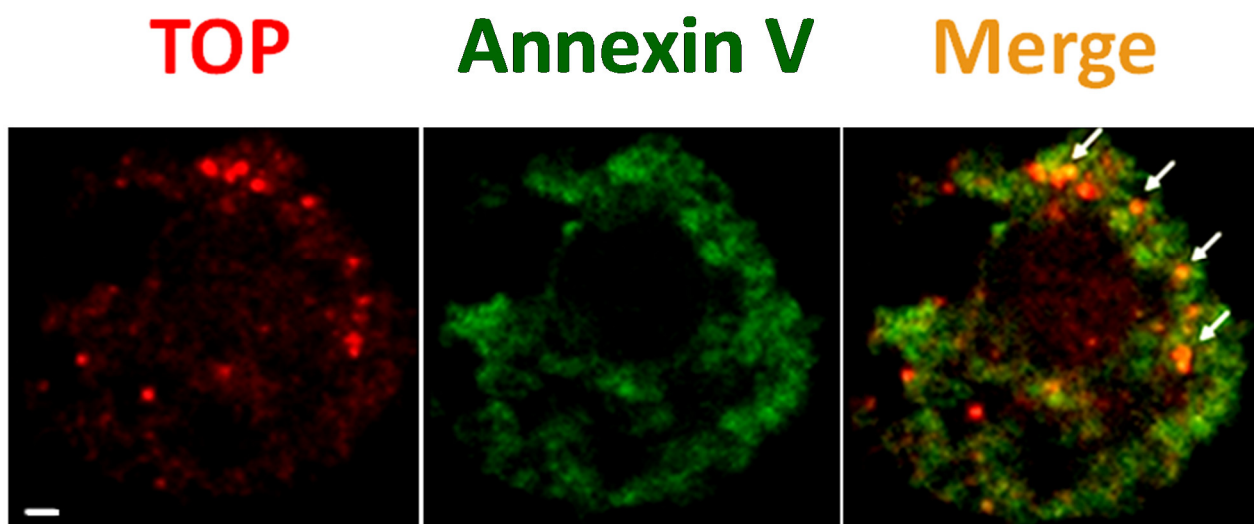
##### **5. Membrane-Associated TOP, and the Novel Membrane Vesicle-Associated TOP in the Extracellular Space**

As noted, although a major portion of peptide processing is initiated in the intracellular space, many peptides are further modified and activated in the extracellular milieu by enzymes like TOP [2,4]. Thus, TOP must be localized extracellularly in addition to its intracellular form, either released to the extracellular space as a soluble protein to the extracellular space or associated with the cell plasma membrane on the membrane surface. TOP has also been found in various subcellular locations depending on cell type, including nuclear [49,50], cytosolic, secreted forms [41,42,44,49,50,65,107], and membrane-associated forms [41,42,108], depending on cell types. Acker et al. found that about 20–25% of the total TOP enzyme activity is associated with membrane fractions [41]. Interestingly, TOP has been visualized on the membrane surface of the cell plasma membrane by confocal microscopy [108]. In addition, TOP is secreted to the extracellular milieu [2,4,65,107], and TOP activity has been detected in the media of DHT-treated LNCaP androgen responsive cells [46].

Extracellular vesicles (EVs) are small subcellular membrane blebs which are released from almost all cell types upon cell activation or during various types of programmed cell death, such as apoptosis, pyroptosis, necroptosis, and NETosis [109–117]. EVs can be generally classified into three main subgroups based on their size and subcellular origin: exosomes (<100 nm), microvesicles (MVs) (<1 µm), and apoptotic bodies (1–5 µm) [112,116,118,119]. Exosomes are the smallest EVs by size (30–100 nm in diameter) and are secreted from the intracellular space [116]. Membrane microvesicles (MVs)

range from 100 to 1000 nm in diameter and bud directly from the cell plasma membrane surface [112,118,120]. Importantly, EVs carry a vast array of intracellular or membrane-associated molecules, such as a variety of biologically active proteins, membrane receptors, lipids, and nuclear DNA or small RNAs from the nucleus, cytoplasm, and cell membranes [109–112,116,121]. EVs are released by almost all eukaryotic cells [112,118] as well as bacteria [122]. They can serve as shuttles which transfer cargos from parental cells to recipient cells that are either nearby or far away [112,116,118,121,123]. In this way, EVs may modulate the function of target cells in response to physiological or pathological stimuli. In contrast to classical chemical signaling pathways, EVs provide alternative modes of paracrine and endocrine communication via direct cell-to-cell contact or receptor–ligand mediated signaling [116]. EVs have been associated with various disease processes. In fact, EV levels have been shown to be elevated in metabolic diseases, infections, and malignancy [116,124–127].

Our previous work showed that matrix metalloproteinase superfamily 14 (MMP14), a transmembrane protease, can be released with EVs when macrophages are stimulated by tobacco smoke extract, and the EV-associated MMP14 is biologically active for collagen cleavage [109]. The works from our and other groups have shown that TOP could be visualized on the cell plasma membrane surface by confocal microscopy [108,113]. Interestingly, we also found that membrane-associated TOP can also be released with EVs (Figures 2 and 3) [113], in addition to its soluble form [46], to the extracellular space. We showed that TOP-positive EVs are generated from cultured DU145 prostate cancer cells that were treated without or with dihydrotestosterone, and that TOP-positive EVs are also generated by PC3 prostate cancer cells when stimulated by calcium ionophore A23187 (Figure 3) [113]. Interestingly, EV-associated TOP exhibits considerable enzymatic activity as measured via quenched fluorescence assay [113]. These results suggest that EV-associated TOP might be a previously unrecognized, novel form of extracellular TOP [113]. It is unknown whether the release of EV-associated TOP from the plasma membrane is a regulated process or a random consequence of cell activation or cell death, making this an area worthy of further investigation.



**Figure 3.** TOP-positive EVs bud from the plasma membrane surface of A23187-activated cells. PC3 prostate cancer cells were treated with 10  $\mu$ M calcium ionophore A23187 for 24 h. Cells were then chemically fixed and detected with anti-TOP antibody (red, labeled as “TOP”) and FITC-labeled “Annexin V” (green, which preferentially binds externalized phosphatidylserine). The yellow color in the merged images (“Merge”) demonstrates co-localization of the two labels, which represents TOP-positive EVs. Scale bar 1  $\mu$ m.

In considering the above findings, one may ask why TOP is found on EVs and what potential advantages this association with EVs may confer to the enzyme. Many physio-

logical substrates of TOP, such as bradykinin, GnRH, and neurotensin, are found either in the extracellular milieu or in the circulation. In order to regulate its physiological substrates, TOP must be accessible to the extracellular space. Previous studies have shown that TOP can be secreted to the extracellular surroundings as a soluble free protein [2,65,107]. Studies of cytokines, which are small proteins that are secreted by immune cells, have short half-lives in the circulation (typically <1 h) [128,129]. Notably, cytokines on the surface of cancer cell derived EVs remain functional and unchanged at 24 h following incubation in plasma at 37 °C, demonstrating that association of cytokines with the EV surface profoundly increases their stability [130]. Icimoto et al. reported that free protein of recombinant TOP is not catalytically stable, while efficient immobilization of TOP in gold nanoparticles can significantly increase stability of the enzyme [131]. Therefore, one may expect that EV-associated extracellular TOP may be more stable in its catalytic activity [113], thus making it a more potent mediator of substrate function [132]. Thus, EV-associated TOP might be more physiologically meaningful than soluble TOP in the pathophysiology of human diseases.

Extracellular vesicles were shown to be important in cancer metastasis [133]. Therefore, EV-associated TOP may be particularly important in cancers of tissues such as that of the prostate. In fact, a number of TOP-sensitive substrates in the proliferation of prostate cancer cells represent an interesting area of study in the field of cancer research. As early-stage prostate cancer proliferation is dependent on androgens, early-stage prostate cancer can thus be effectively treated with androgen-ablation therapy. Beginning in the early 1980s, analogs of TOP substrate GnRH-I had already been used in therapeutic approaches against prostate cancer [134,135] by suppressing the hypothalamic–pituitary–gonadal axis during the early androgen dependent phase. Recent studies have shown that GnRH in fact decreases prostate cancer cell growth [134]. As a crucial modulator of the activities of GnRH and its other physiological substrates, extracellular TOP stands as an important subject of study in further understanding the pathophysiological significance of these hormones in the role of prostate cancer disease progression. Our findings demonstrate a new manifestation of extracellular TOP as being carried by EVs when released from prostate cancer cells [113]. In contrast to the soluble form of TOP, which easily diffuses into the circulation and can be diluted by the large volumes of blood in the circulation, EV-associated TOP may be retained in the tumor microenvironment in relatively high concentration [113,116]. Therefore, the EV-associated format of TOP may enable the enzyme to work more potently on its substrates within a microenvironment, thus contributing to the progression of pathological conditions.

In addition to TOP, there are other cell membrane peptidases that are involved in the inactivation or activation of extracellular hormones, cytokines, regulatory peptides, paracrine peptides, and neuropeptides [136]. A notable peptidase is dipeptidyl peptidase IV (DPPIV), which is also a cell surface peptidase [136]. Kandzija et al. recently reported that placental EVs can carry bioactive DPPIV and these DPPIV-positive EVs are elevated in patients with gestational diabetes mellitus [137]. The EV-associated bioactive DPPIV can cleave glucagon-like peptide-1, which is known to regulate glucose-dependent insulin secretion and, therefore, contributing to diabetes [137]. Furthermore, ACE2 is a carboxypeptidase which has also been localized to the cell membrane surface. Several recent studies [89,138–140] have reported that ACE2 can be released with EVs (EVs and exosomes), and that these ACE2-associated EVs can competitively bind SARS-CoV-2 virus spike protein. In turn, EV-carried ACE2 may serve as a novel therapeutic strategy for COVID-19 prevention and/or treatment [139]. All in all, there is now direct evidence of at least three peptidases (TOP, DPPIV, and ACE2) which can be associated with EVs. Therefore, EV-harboring peptidases might be a common natural phenomenon. Whether other peptidases are associated with EVs would be a worthwhile area for further investigation [137].



## 6. Conclusions

Thimet oligopeptidase (TOP) has classically been considered a cytoplasmic enzyme whose substrates are mainly extracellular peptides. Later studies revealed an intracellular role for TOP. Our results indicate that TOP is released on extracellular vesicles from prostate cancer cells, consistent with findings concerning peptidases on EVs from other tissues.

Taken together, the wide range of locales and substrates for TOP suggests potential for the enzyme's role in regulation and treatment of a variety of natural and pathological states.

## 7. Summary

Since TOP was first characterized over 40 years ago, its roles in processing peptides in the extracellular and intracellular space have been elucidated with biochemical, cellular, and molecular approaches, for its potential involvement in neuroendocrinology, behavior, metabolic and cardiovascular diseases, cancer, and infectious diseases. Novel findings about membrane-associated TOP may provide insights for potential diagnosis and therapeutic strategies in a wide variety of diseases.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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