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REVIEW

Trypsinogen and chymotrypsinogen: potent anti-tumor agents

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ABSTRACT

Introduction: Trypsinogen and chymotrypsinogen have been used clinically in tissue repair due to their ability to resolve inflammatory symptoms. Recently, novel evidence has supported the anti-tumourigenic potential of a mixture of trypsinogen and chymotrypsinogen.

Areas covered: First, we analyze the structure of these proteases and the effects of pancreatic proteinases on tissue repair, inflammation and the immune system. Second, we summarize studies that provided evidence of the effects of pancreatic (pro)enzymes on tumor cells both *in vitro* and *in vivo* and some successful clinical applications of pancreatic (pro)enzymes. Finally, we study pancreatic (pro) enzymes potential molecular targets, such as the proteinase-activated receptors (PARs).

Expert opinion: This novel therapy has been shown to have effective antitumor effects. Treatment with these (pro) enzymes sensitizes Cancer Stem Cells (CSCs) which may allow chemotherapy and radiotherapy to be more effective, which could positively affect the recovery of cancer patients.

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Cancer; Chymotrypsin; pancreatic enzymes; proteinase-activated receptors; Trypsin

1. Introduction

Despite the latest advances in recent years, the incidence of cancer is continuously increasing [1]. Currently, the basis of cancer therapy relies on radiotherapy and chemotherapy notwithstanding their undesirable side effects. For this reason, new less invasive treatments are being investigated with the aim of improving the anti-tumor efficiency while reducing the side effects of current treatments [2].

The pancreas plays a very important role in the digestive function through the secretion of several enzymes necessary for the degradation of nutrients. These enzymes are secreted by acinar cells as zymogens (inactive forms also known as (pro)enzymes) [3]. Once secreted, they are transferred to the small intestine where they are activated. The most studied zymogens are Trypsinogen and Chymotrypsinogen. In the case of Trypsinogen, it is activated to Trypsin in the small intestine by enterokinase. Once activated, it is capable of activating the rest of the pancreatic zymogens, including Chymotrypsinogen into Chymotrypsin [4]. A failure in the production of these proteins can cause poor absorption of nutrients, the most common diseases that lead to exocrine pancreatic insufficiency are chronic pancreatitis and cystic fibrosis [5].

Both (pro)enzymes belong to the serine protease family and, although their structure is quite similar, there are some key differences between them. One of the most relevant disparities resides on the amino acids that constitute their active centers, which confers the (pro)enzymes different substrate affinity [6]. Furthermore, it has been reported that there are different isoforms with distinct functions [7,8].

Studies in patients suggest that Trypsin and Chymotrypsin have an important role in tissue regeneration showing a positive anti-inflammatory effect [9]. It also appears that these proteins may have an effect on increasing the activity of cells of the immune system [10]. Also, there are several *in vitro* and *in vivo* studies reporting the anti-tumor potential of pancreatic (pro)enzymes [11]. Although the antitumor effects of those proteins are clear, their mechanisms of action are currently under investigation.

Here, we will examine the structure of Trypsinogen and Chymotrypsinogen with the aim of understanding how pancreatic (pro)enzymes have such a wide range of effects. Then, the possible implications of these (pro)enzymes along with their different isoforms regarding the use of pancreatic enzymes as anti-cancer therapy will be discussed. In addition to this, we will review the most recent clinical use of the pancreatic (pro)enzymes as an alternative therapy for diverse medical conditions. Next, we will review several published studies involving patients reporting a slight beneficial effect of the pancreatic (pro)enzymes on cancer therapy. Finally, we will discuss the potential molecular targets of these pancreatic (pro)enzymes putting special emphasis on the proteinase-activated receptors (PARs).

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Article highlights

- Trypsin and chymotrypsin have anti-inflammatory effects and are capable of increasing the activity of the immune system.
- In vitro, in vivo and clinical studies suggest an antitumor effects of Trypsinogen and Chymotrypsinogen.
- The (pro)enzymes cause re-differentiation of tumor cells.
- (Pro)enzymes cause a reduction in the population of CSCs, impairing their pluripotent phenotype, their metastatic capacity and the epithelial-to-mesenchymal transition.
- (Pro)enzymes affect the TGF-beta pathway.
- The possible molecular targets of these (pro)enzymes may be the proteinase-activated-receptors (PARs).
- Trypsin has a preference to activate PAR-2, while Chymotrypsin mainly activates PAR-1.

This box summarizes key points contained in the article.

2. Trypsinogen and Chymotrypsinogen: structural elements

Trypsinogen and Chymotrypsinogen belong to the serine protease family which is mainly composed of hydrolases [12]. The structure of Trypsinogen/Trypsin and Chymotrypsinogen/Chymotrypsin is very similar and consists of two beta-barrels with eight loops around the active site. The active site is found between these barrels and is composed of three amino acids. The first two correspond to His-57 and Asp-102 that belong to the N-terminal beta-barrel, whereas the other amino acid Ser-195 comes from the C-terminal barrel (Figure 1). In addition, the proteins are fairly stabilized by six disulfide bridges to resist the reducing environment in the gastrointestinal tract [13]. Although Trypsinogen and Chymotrypsinogen have similar tridimensional structures, the substrate specificity of their active sites is different. The difference in the active sites is the result of the distinct residues that form the binding pockets. Whereas Trypsin has an aspartic acid, Chymotrypsin has a serine which produces specificity for aromatic residues [14]. The active site of Trypsin is specific for arginine and lysine residues, while Chymotrypsin is specific for aromatic residues like tyrosine, phenylalanine and tryptophan [6]. Therefore, the differences between the two enzymes rely on the breaking down of different amino acids and how they can consequently digest different proteins. Furthermore, their different substrate specificity also promotes the activation of different molecular targets which may imply the activation of different molecular pathways and the promotion of distinct biological effects (see below).

Trypsinogen is activated to the proteolytic enzyme Trypsin in the duodenum by enterokinase (Figure 2) which recognizes the specific sequence in the propeptide of Trypsinogen, Asp-Asp-Asp-Asp-Lys, and cleaves after the Lysine residue in the peptide bond Lys-23 and Ile-24 [15]. Other proteinases, such as matrix metalloproteinase-9 and cathepsin B, activate Trypsinogen but

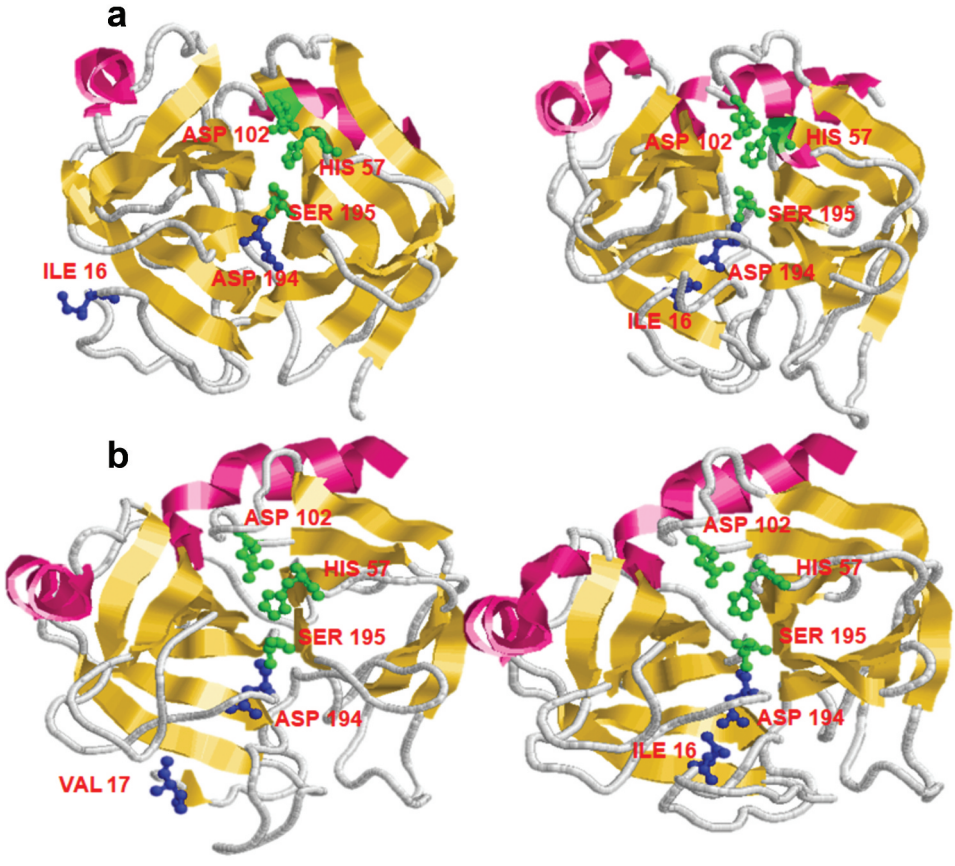

Figure 1. General structure of Chymotrypsinogen/Chymotrypsin and Trypsinogen/Trypsin represented in ribbon and in color. a) The structure of Chymotrypsinogen (zymogen) is shown on the left and the structure of Chymotrypsin (active form) on the right. b) The structure of Trypsinogen (zymogen) is shown on the left and the structure of Trypsin (active form) on the right. The amino acids that make up the active site are shown in green. In the case of the active structures in blue, the interaction between the amino acids Ile-16 and Asp-194 is shown, which are involved in the formation of a salt bridge necessary for the activation of proteins.

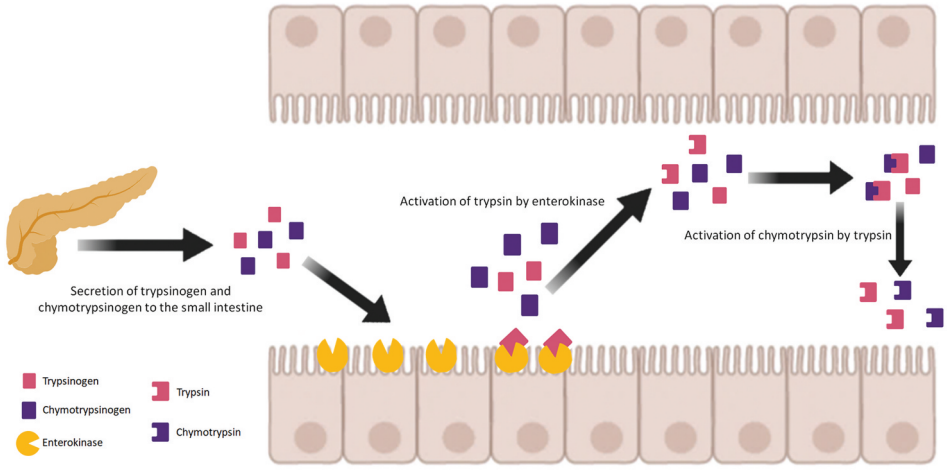

Figure 2. Activation mechanism of proenzymes in the small intestine.

are related to acute pancreatitis [16,17]. Once the activation peptide is removed, a conformational change is observed in the protein structure which allows the substrate attachment in the specific pocket [18]. After Trypsin activation, in the 70–80 loop, three amino acids Glu-70, Glu-72 and Glu-80 bind Ca^{2+} increasing the stability of the active protein [19], so it is well within reason to assume that the Ca^{2+} concentration may condition Trypsin half-life and hence its biological activity.

Trypsin, in turn, activates Chymotrypsinogen, but in this case, the propeptide does not dissociate. In fact, the propeptide of the Chymotrypsinogen remains linked to the rest of the enzyme via a disulfide bridge in the activated forms of these proteins [20].

Following, another important process occurs in the activation process of these proteins. In the 189-loop, a salt bridge is formed between the N-terminal of the residue Ile-16 (which was previously linked to the activation propeptide) and residue Asp-194, leaving the Ser-195 of the catalytic site free [21]. Considering how the amino acids involved in the active site are not consecutive in the primary amino acids sequence but are spatially near in the tridimensional structure, the correct folding during the protein synthesis may be essential. Furthermore, the structural changes after the cleavage of the propeptides suggest the importance of cleaving the (pro)enzymes in a specific site in order to promote the right conformational changes leaving the catalytic center accessible. Interestingly, a study that used the Gaussian network model and a clustering method to analyze the dynamic properties of Trypsin and Chymotrypsin has suggested that the cooperative motions of the two loops and the substrate-binding sites contribute to the activity and substrate specificity of Trypsin and Chymotrypsin. Thus, sites that are spatially distant from active sites can have a strong mechanical influence on the structural modulation of the substrate-binding regions [6]. These facts may partially explain the benefits of using (pro)enzymes rather than the already activated enzymes since it has been reported that tumor cells can activate the (pro)enzymes through a protein that differs from enterokinase [22].

Additionally, the human pancreas secretes different isoforms of Trypsinogen and Chymotrypsinogen. Three different isoforms of Trypsinogen: cationic isoform, anionic isoform and Mesotrypsinogen have been described. The prevalent form is the cationic isoform followed by the anionic isoform and finally the Mesotrypsinogen that represents less than 5% [7,23]. Cationic and anionic Trypsinogen have similar characteristics with respect to their molecular weight, amino acid composition, and optimal pH [24]. The differences between anionic and cationic isoforms involve the ability of cationic isoforms to autoactivate at an acidic pH and the higher stability of cationic Trypsinogen. On the other hand, the anionic isoform autolyzes itself faster at neutral or alkaline pH [25]. In addition, calcium ions are unable to stabilize the anionic isoform against autolysis [24]. The enzyme Mesotrypsin is characterized by its resistance to trypsin inhibitors and for promoting their degradation [26]. Specifically, it has been reported that this resistance is due to the presence of an arginine instead of glycine at position 198 [27]. Regarding Chymotrypsinogen, four different isoforms have been described: Chymotrypsinogen/Chymotrypsin B1, Chymotrypsinogen/Chymotrypsin B2, Chymotrypsinogen/Chymotrypsin C and Chymotrypsin-like protease. Chymotrypsin B1 has a preference for amino acids like Tryptophan and Tyrosine, while Chymotrypsin B2 has a preference for amino acids such as Phenylalanine and Tyrosine [28]. Chymotrypsin C preferentially cleaves the peptide bonds located in the C terminal of tyrosine, methionine and leucine and it can activate Trypsinogen by cleaving at the activation peptide between Phe-18 and Asp-19 residues [15]. Conversely, it can also cause the degradation of Trypsin by cutting into the calcium-bindingloop between the Leu-81 and Glu-82 residues which bind to Ca^{2+} to stabilize the protein resulting in a rapid degradation and inactivation of cationic trypsinogen. This may be a regulatory mechanism when trypsinogen is overexpressed or activated too early [29]. The specific cleavage of the Leu81-Glu82 peptide bond in human cationic trypsinogen by CTRC is primarily determined by its distinctively high activity on leucyl peptide bonds, with the P1′ Glu82, P3′ Asn84 and P4′ Glu85 residues serving as additional specificity determinants [28]

Chymotrypsin-like protease has a 56% identity to Chymotrypsin B, and different substrate specificity because this enzyme hydrolyses at Tyrosine, Leucine or Phenylalanine [30].

Importantly, most of the studies showing the anti-tumor effects of enzyme therapy cited below used pancreatic extracts that might contain all the isoforms described here. Therefore, it is reasonable to suggest that the beneficial therapeutic effects of the (pro)enzymes may be a result of the combined participation of all isoforms. Furthermore, cationic Trypsinogen is self-activated only at an acidic pH which may be relevant considering the acidic nature of tumor microenvironment compared to the neutral pH of healthy tissues [31]. Therefore, it could be suggested that the cationic Trypsinogen may be a key role in the anti-tumor effects of pancreatic (pro) enzymes against cancer cells described below.

3. Effects of pancreatic proteinases on tissue repair, inflammation and the immune system

One of the successful therapeutic uses of the Trypsin and Chymotrypsin combination involves tissue repair. In fact, both proteins have been used for treatment of surgical or accidental injuries, fractures, and burns since the 1960s [32]. Moreover, several studies have shown the potential of pancreatic enzyme combination as an anti-inflammatory, anti-edematous, fibrinolytic, antioxidant and anti-infective agent and to have an analgesic effect [9].

Oral combination of Trypsin:Chymotrypsin targets early stages of inflammation. It appears that the high affinity of α1-antitrypsin for Trypsin and Chymotrypsin compared to plasmin leaves plasmin available for fibrinolysis shortening the period of fibrinolysis. This causes the reduction of clots in the blood vessels, restoration of circulation, a decrease in inflammation and edema, and consequently favors the regeneration of the damaged tissue [33]. Other studies have been conducted in patients with orthopedic surgery or sciatica who were treated with the combination of Trypsin:Chymotrypsin. Recovery time in treated patients decreased, and no side effects were reported [32]. In addition, burn patients treated with Trypsin:Chymotrypsin showed a rapid recovery and a decrease in inflammation [34].

In an immune context, it has been shown that Trypsin increases the phagocytic activity of macrophages, thus preventing the wound from becoming infected [32]. In agreement, Trypsin and Chymotrypsin treatment enhances macrophages phagocytic activity and decreases infection rate in patients [32]. Interestingly, some studies reveal that oral proteolytic enzymes are able to directly stimulate the activity of immune competent cells, suggesting that the proteolysis of intestinal microorganisms stimulates immunocompetent cells [10]. In fact, microbial fragments, such as lipopolysaccharides, muropeptides, β-glucans, etc., are well known for their immunostimulatory action [35]. Moreover, proteolytic enzymes have a high affinity for α 1-antitrypsin and α 2-macroglobulin. The interaction between proteinases and those proteins affects other cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interferon-γ and TGF-β influencing the immune response [36].

These effects of pancreatic enzymes may reinforce their anti-tumor potential since cancer has been defined as an inflammatory disease. In addition, it is well known that cancer cells commonly escape from immune surveillance and that has potent immunosuppressive effects leading to the expansion and aggressiveness of the cancer, and thus, enzyme therapy may counteract said advantage held by cancer.

4. Anti-tumor effects of pancreatic proteinases

Recapitulating from the previous section pancreatic (pro) enzymes have an active role in inflammatory process attenuation and in immune system potentiation, both critical events in a tumourigenesis setting. In fact, the therapeutic use of serine endoproteinases, Trypsin and Chymotrypsin (extracted from either porcine or bovine pancreatic juice), and the plant cysteine endoproteinases, bromelain and papain, against cancer has been successfully tested [11].

Several published studies involving patients have evaluated the efficacy and/or safety of pancreatic enzyme therapy. Table 1 summarized reported studies based on the administration of pancreatic (pro)enzymes to oncological patients. These studies included more than 1600 patients that have been treated with pancreatic enzymes for up to 7 years. Treatment with pancreatic enzymes increased the survival rate, decreased the recurrence of the disease and the frequency of metastasis in many types of malignancies such as pancreatic adenocarcinoma [37], cancer of the uterine cervix [38], head and neck cancer [39], colorectal cancer [40], pancreatic cancer [41] multiple myeloma [42] and breast cancer [43,44]. Furthermore, enzyme tolerability was good and showed no adverse effects. Nevertheless, although the results showed, in general, a positive impact on the treatment of cancer patients, some limitations due to studies that were not well-designed and the lack of double-blinding in some cases should be noted. For instance, Gonzalez and Isaacs [37] found that patients treated with pancreatic enzyme therapy exhibited an increased time survival. However, this study was only conducted with a small number of patients, and there was no control group for comparison. Dale et al. [38], showed that the treatment helps to reduce the symptoms of radiotherapy when used in an adjuvant manner, but the study lacked double-blinding and did not analyze the effect of the sole treatment on patient survival. In addition, others have shown that pancreatic enzymes could reduce the undesired symptoms of chemotherapy and radiotherapy, but the relatively short time frame of the trials may reduce the relevance of the findings [39,40]. On the other hand, a better designed study conducted by Sakalová and colleagues [42] showed a significant difference between survival time of patients with stage III multiple myeloma who were treated with enzymes and patients who did not receive enzymes, 83 months versus 47 months, respectively. In this respect Beuth et al. [43], and Peran et al. [45], also observed improved time survival in tumor patients treated with pancreatic enzymes. Finally, it is interesting to comment on the conclusión of the study reported by Chabot and colleagues who compared gemcitabine-based chemotherapy with enzyme therapy for the treatment of pancreatic cancer, 'Among patients who have pancreatic cancer, those who

Table 1. Adjuvant proteolytic enzyme therapy in studies involving patients.

Trial Design/ Cancer Type/ No of patients/ Reference	Adjuvant Proteolityc Enzyme therapy/ Treatment Duration	Main Results
Unblinded- single-arm Adenocarcinoma Treated group: 11 [37]	25–40 g of porcine lyophilized pancreas product 2 years	Increased survival rate in the treated patients (17 months)
Retrospective cohort, with parallel groups Non-metastatic Breast cancer Control group: 1056 Treated group: 1283 [43]	Mixed proteolytic enzymes papain, trypsin and chymotrypsin (Wobe Mugos E) 485 days	Reduction of radiation therapy/chemotherapy side effects. Reduction of the disease-associated symptoms. Metastasis and recurrence decreased in treated patients.
Retrolective cohort analysis with parallel groups colorrectal cancer Control group: 626 Treated group: 616 [40]	Mixed proteolytic enzymes papain, trypsin and chymotrypsin (Wobe Mugos E) 9.2 months	Increased survival of treated patients (34.1 months). Reduction of the disease-associated symptoms
Retrolective cohort analysis, with parallel groups Multiple myeloma Control group: 99 Treated group: 166 [42]	Mixed proteolytic enzymes papain (100 mg), trypsin (40 mg) and chymotrypsin (40 mg) (Wobe Mugos E) 61 months	Increased survival of treated patients (3 years). Good proteolytic enzyme therapy tolerability
Prospective randomized open study Uterine cervix cancer Control group: 60 Treated group: 60 [38]	Radiation therapy + mixed proteolytic enzymes papain, trypsin and chymotrypsin (Wobe Mugos E) Less than 2.5 months	Reduction of radiation therapy side effects
Prospective randomized open study Head and neck cancer Control group: 47 Treated group: 43 [39]	Gamma-radiation + mixed proteolytic enzymes papain (100 mg), trypsin (40 mg) and chymotrypsin (40 mg) (Wobe Mugos E) Less than 8 weeks	Reduction of radiation therapy side effects
Non randomized study Breast cancer Treated group: 57 [44]	Chemotherapy + mixed proteolytic enzymes papain, trypsin and chymotrypsin (Wobe Mugos E) One year	Good proteolytic enzyme therapy tolerability
Non randomized controlled study Pancreatic cancer Control group: 23 Treated group: 32 [41]	Pancreatic enzymes, nutritional supplements, detoxification and organic diet 40 months	No effect was seen in the group of those treated with enzymes
Non randomized study Several types of cancer Treated group: 46 [45]	Suppository formulation containing 8.92 mg of each bovine pancreatic pro-enzymes (Trypsinogen and Chymotrypsinogen A) and 1.78 mg of α-amylase 9 months	Increased survival of treated patients

chose gemcitabine-based chemotherapy survived more than three times as long (14.0 v 4.3 months) and had a better quality of life than those who chose proteolytic enzyme treatment' [41]. Therefore. this study corroborates the idea that enzyme therapy must be understood as an adjuvant treatment that could improve conventional cancer treatments. In addition, despite the potentially positive outcome of the clinical studies described above, there is still room for improvement regarding enzyme-therapy. These enzymes had to be administered in high quantities to ensure the absorption of intact proteins into the blood circulatory system [37] and controversial results have arisen over the amounts of enzymes that actually bypass the gut barrier. For instance, some studies showed no absorption into blood of pancreatic enzymes after oral administration [46], with the remainder being digested in the gastro intestinal tract. While other authors suggest that these enzymes are acid stable and are absorbed through the gastrointestinal mucosa and into the blood stream as part of an enteropancreatic recycling process [47]. In conclusion, to avoid a high dosage of enzymes administration and its possible reduced oral absorption, other alternatives, such as intravenous administration, should be considered. By administering a proenzyme mix by intravenous injection, digestion of the proenzymes in the duodenum will be avoided and they will be absorbed intact reducing the dose quantities used historically for these therapies. In fact, when a formulation of Trypsinogen and Chymotripsinogen was administrated intravenously to mice it was shown that tumuor initiation diminished in animals injected with pancreatic derived cancer stem cells, showing in vivo an effect of pancreatic pro-enzymes on CSCs engrafting [48]. Other studies with animals models also confirmed the anti-tumor effect and anti-metastatic effect of proteolytic enzyme treatment. Positive results were found in a vast variety of tumors induced in mice, such as melanoma [49,50], Lewis lung carcinoma [51], mammary tumor [52], ovarian cancer [45] and pancreatic cancer [45,48,53]. Table 2 summarizes the main results on these studies suggesting a great potential of these digestive proteases as an alternative anti-cancer therapy.

The studies summarized above, both in the clinical *arena* and in the *in vivo* settings, were performed with a variety of proteolytic enzyme/(pro)enzyme combinations which include bromelain, papain, amylase, lipase, trypsin and α-Chymotrypsin. As the effectiveness of a reliable treatment lies in its simplicity, reproducibility and safety, our group conducted a series of experiments with the aim of determining the synergetic combination of pancreatic (pro)enzymes that is the most efficient as an anti-cancer agent. We began by carrying out a blind study comparing the half maximal inhibitory concentrations (IC50) of different pancreatic (pro)enzyme/enzyme combinations and selected the combination of Trypsinogen and Chymotrypsinogen. Further, we implemented an extensive study *in vitro* evaluating growth inhibition, in 24 different human cancer cell lines, and determined 1:6 as the optimal ratio of the Trypsinogen and Chymotrypsinogen combination [45,54]. The formulation comprising of the two pancreatic (pro)enzymes Trypsinogen and Chymotrypsinogen was named as PRP and was further validated through different studies and assays following a designed strategy to evaluate the utility of this therapy in advanced solid tumors. Interesting results were obtained after treatment of Caco-2 with PRP which induced enterocytic differentiation [54]. Cells acquired a polarized structure with an enterocyte-like phenotype characterized by the appearance of cell-specific differentiated structures such as microvilli and tight junctions, which is in agreement with other studies showing that pancreatic Trypsin induces tight junction or dome formation in some colon cancer cell lines (HT-29, Caco-2) [55]. In addition, cell differentiation induction by the (pro)enzyme formulation was also proven in the pancreatic tumor cell-line Panc-1. Treated Panc-1 cells aggregated and formed islet-like clusters that are characteristic of pancreatic cell differentiation [56]. Others studies also support the formation of cellular aggregates similar to multicellular spheroids after Trypsinogen and Chymotrypsinogen treatment and, furthermore, they showed the benefits of using the (pro)enzymes rather than the active enzymes, since (pro)enzymes may be activated specifically on tumor cells but not on healthy ones [22]. Moreover, differentiation of Panc1 cells was further confirmed by an increment on lamellar bodies and an increased expression of pancreatic differentiation-related markers [54]. Pancreatic enzymes may thus enforce the reentry of tumor cells into the normal pathways, thus acquiring a less malignant phenotype and a reduced proliferative capacity due to lineage specific cellular differentiation [54]. Further, the antiangiogenic potential of PRP was analyzed by matrigel-based tube formation and by fibrous capsule formation assays, showing that treatment decreased the number of capillary-like structures [45].

The anti-cancer effects of PRP formulation were also tested on pancreatic cancer stem cells (CSCs), tumor-initiating cells that have quiescent capacities and are thought to be responsible for metastasis [57]. It was observed that the treatment reduced the CSC population measured by the expression of CSC-related markers like ALDH [58], CD44, CD326 and CXCR4 [59]. In addition, PRP treatment induced the downregulation of relevant EMT-related genes and promoted an increase in the cell adhesion, cell differentiation, and expression of tumor suppressor genes [48]. Thus, the (pro)enzyme treatment inhibited the expression of genes related to the CSC phenotype, changing the nature of these malignant cells toward a more differentiated and less dangerous cellular condition. The mentioned cascade of reactions induced by the (pro)enzyme treatment is summarized in Figure 3. In short, PRP treatment may promote the upregulation of RAC1β which may prevent the hyper-activation of the TGF-β pathway. This may prevent the TGF β-induced p38 pathway leading to the phosphorylation of YAP which may inhibit the Notch pathway. In addition, phosphorylated YAP sequesters β-catenin in the cytoplasm which may block the canonical Wnt pathway [48]. Those events implied the inhibition of the EMT phenomenon, a known process which leads to CSC phenotype and metastatic dissemination. In fact, the loss of differentiation-associated features and the concomitant loss of cell-cell contacts after the EMT process strongly enhance migration and invasion of the surrounding tissues leading to metastasis [60].

Table 2. Summary of *in vivo* assays with proteolytic enzyme treatments.

Animal model/ Tumor cells/ Duration of treatment/ Reference	Treatment	Main results
Female mice C57Bl6 Melanoma cells (B16) 100 days [49]	45 mg/kg of the mixed proteolytic enzymes papain, trypsin and chymotrypsin (Wobe-Mugos E) rectally administered, twice daily.	Survival rate increased in the treated group (58.3%). Metastasis decreased in treated mice.
Female C57Bl6 mice Lewis lung carcinoma cells 100 days [51]	0.1 mL saline solution containing 0.5 mg papain, 0.5 mg trypsin and 0.2 mg chymotrypsin, rectally administered, twice daily. Treatment commenced: (A): after extirpation; (B): 6 days before extirpation; (C): inmediately following transplantation	Survival rate increased in the treated groups (A: 60%; B: 90% and C: 100%). Metastasis decreased in treated mice.
Female F-344 rat Rat mammary tumor (R13762) cells 50 days [52]	20 g or 2 g porcine pancreatic extract (2 groups for each condition) + 10 mg Mg++ daily (only one group for each condition). Orally administered, daily.	No differences in the tumor growth were observed between the treated groups and control group. Also, no differences in the incidence of rats with pulmonary metastasis.
Female C56Bl6 mice Melanoma cells (B16) 100 days [50]	0.1 ml of 0.2 mg trypsin, 0.5 mg papain and 0.2 mg chymotrypsin rectally administered, twice daily. Treatment commenced: (E1): 24 h after intracutaneous transplantation; (E2) after extirpation of the primary Bl6 melanoma	Treated E1 group showed no tumor formation (36%) and small size of the tumor. Survival increased in treated groups (E1: 46% and E2: 30%). Metastasis and recurrence decreased in treated mice.
Male beige XID nude mice Pancreatic cancer cells (AsPC1) 60 days [53]	400 mg/kg of porcine pancreatic extracts, Orally administered, daily.	Survival rate increased in the treated group (79%). Tumor size decreased in the treated mice. No reported side effects
Female athymic Nude-Fox1^{nu} mice and female C57BL/6 mice Human ovarian cancer cells (A2780) and mouse pancreatic tumor cells (Pan02) One month [45]	Ovarian tumor: 27.5/165 mg/kg (Group 2); 83.3/500 mg/kg (Group 3). Pancreatic tumor: 9.1/54 mg/kg (Group 2); 27.5/165 mg/kg (group 3). Intravenously injected, 3 days per week.	Tumor weight in the ovarian cancer mice treated with 83.3/500 mg/kg decreased. Tumor weight in the pancreatic cancer mice treated with both doses decreased.
NSG imminodeficient mice Pancreatic cancer (BxPc3 cancer stem cells) 3 months [48]	83.3/500 mg/kg Trypsinogen/Chymotrypsinogen A Intravenously injected, 3 days per week. Pre-treatment group: injections started 3 weeks before tumor induction. Pre-treatment+treatment group: injections started 3 weeks before tumor induction and followed for 9.5 weeks after tumor induction.	Tumor incidence decreased in treated groups. (41% in the pre-treated mice and 50% in the pre-treated+treatment group). Tumor growth decreased in treated mice.

Therefore, the effects of (pro)enzymes by inducing cell differentiation, impairing angiogenesis, inhibiting the stem-like phenotype and blocking the EMT process could represent an advance in the treatment of cancer (Figure 4).

It is interesting to comment that some studies described proteases as a malignant growth promoting factor, for instance [61], shows that mesotrypsin promotes malignant growth of breast cancer cells. Nevertheless, it has also been accepted that related proteases can possess contrasting pro-tumorigenic and anti-tumorigenic functions [62]. In fact, human mesotrypsin has been described as a defective human trypsin due to its compromised ability to cleave protein substrates [26,63] with different sequence, steric, and electrostatic features from closely related proteases [27,64]. The structural and functional differences between mesotrypsin and trypsinogen and chymotrypsinogen could explain the contradictory data on the role of proteases in tumor progression. Actually, we reviewed here three completed studies demonstrating the antitumour efficacy of a synergetic combination of the pancreatic pro-enzymes trypsinogen and chymotrypsinogen [45,48,54]

5. Potential targets of pancreatic proteinases

In the previous section, we have detailed the use of proteolytic enzymes for complementary oncology although the mechanistic information available on enzyme therapies is not yet completed, there are some important findings in this regard. A correlation between enzyme therapy and TGF-β was given by Desser and colleagues [36] who studied the benefits of oral administration of a combination of papain, bromelain, trypsin, and chymotrypsin to patients with elevated TGF-β due to rheumatoid arthritis, osteomyelitis, or herpes zoster. Interestingly, the treatment induced a reduction of TGF-β concentration in blood in patients with elevated TGF-β concentration (>50 ng/ml serum), while no changes were found in control patients or patients with normal TGF-β levels [65]. Additionally, it is well accepted that the TGF-β abnormal pathway plays a role in pancreatic cancer [66] consequently being a therapy that influences the normalization of TGF-β pathway and may be a potential treatment to be considered for a multitargeted therapy strategy against cancer [67]. Of relevance, a recent study has shown a close relationship between PAR4 (a potential target of trypsin, discussed below), and the

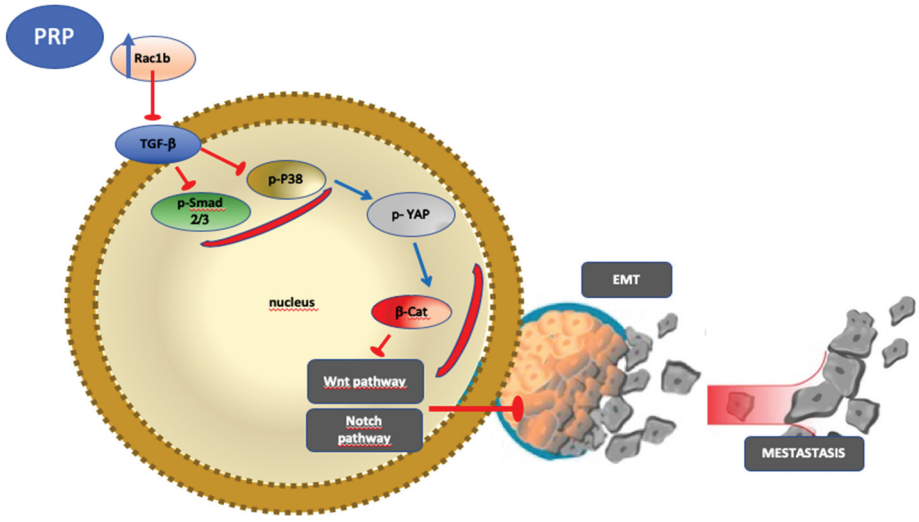

Figure 3. Effect of PRP (Chymotrypsinogen/Trypsinogen) on the molecular mechanism and metabolic pathways of TFGβ induces EMT blocking and metastasis inhibition.

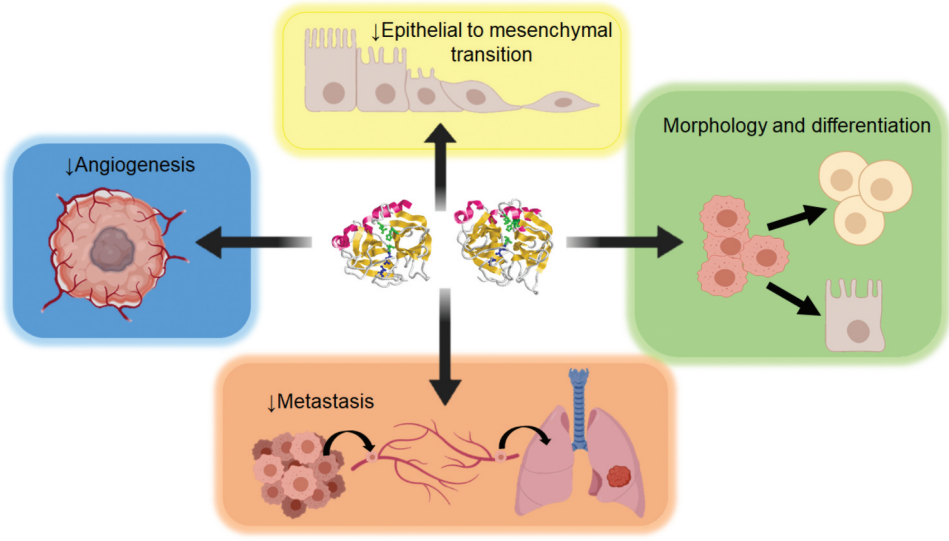

Figure 4. Main anticancer effects of Chymotrypsinogen/Trypsinogen formulation.

upregulation of SMAD4 in pancreatic cancer cells. The authors considered the upregulation of SMAD4 as a key event representing the switch of TGF-β signaling from tumor-supportive to tumor-suppressive pathway [68].

The effect that pancreatic enzymes produce on cancer cells is certainly due to the cleaving of extracellular molecules which triggers intracellular signaling. As proteases, pancreatic enzymes could impair tumor progression by cleaving and disrupting important protein component/structures of the tumor microenvironment matrix such as the 3D collagen architecture, which has been found to promote the migration of tumor cells [69]. However, studies conducted *in vitro*, in which the tumor microenvironment-associated structures are not present, have also shown anti-tumor potential of pancreatic enzymes. In this respect, it has been reported that proteases, like Trypsin or Chymotrypsin, cleave extracellular precursors to generate the active form of several proteins. For instance, Trypsin can cleave pro-insulin to generate the active insulin by a proteolytic mechanism [70,71]. Similarly, other proteolytically activated factors are the epithelial growth factor (EGF) [72], the tumor growth factor A (TGF-A), the pro-tumor necrotic factor A (pro-TNFA) [73,74] and the pro-transforming growth factor beta (pro-TGFβ) [75]. Considering the central role of the proteolytic cleavage in the generation of many active factors/hormones, it seems probable that pancreatic enzymes may act in a larger number of attached/soluble targets than described to date. Apart from this role in proteolytic cleavage that is 'non-receptor' targeted, several membrane receptors which are also susceptible to being cleaved and activated by pancreatic enzymes have also been described. In this context, we look at the insulin-like function of Trypsin since this proteinase is able to cleave and activate the insulin receptor in cell surfaces [76,77]. This fact may be interesting since a relationship between insulin receptors and

several types of malignancies, such as pancreatic cancer [78], prostate cancer [79] or renal carcinoma [80] among others, has been shown. Additionally, the insulin-like growth factor 1 receptor (IGF1R) is also related to insulin and it might be susceptible to being proteolytically activated. Therefore, it could also be a potential target of the digestive enzymes like Trypsin although this fact is still yet to be confirmed. This hypothesis may be relevant considering that a relationship between IGF1R and cancer progression [81], for instance colon cancer [82] or ovarian cancer [83] has been reported.

Finally, a family of 4 G protein-coupled membrane receptors, known as proteinase-activated-receptors (PARs), have been described potential targets for pancreatic proteolytic enzymes [84]. Searching for cellular surface targets of thrombin led to the discovery of the first member of this family of membrane receptors (PAR-1) in early 1990s [85]. Initially, it was named as 'thrombin receptor' but some years later it was known as PAR-1 according to IUPHAR nomenclature [86]. The discovery of this family of receptors (completed with the identification of the other three members PAR2, PAR3 and PAR4) was followed by the study of their activation procedure. The mechanism by which a proteinase (i.e. Trypsin and/or Chymotrypsin) can activate PARs is a well-known process composed of two main steps (Figure 5). First, the proteinase cleaves the receptor extracellular domain in a specific point. This proteolytic cut unmasks a cryptic receptor-activating sequence (also known as 'tethered ligand') in the N-terminal region of the receptor which, remaining attached, binds to the extracellular activating site of the PAR. Second, this binding triggers a conformational change in its structure which promotes the receptor-coupled G protein-dependent intracellular signaling [87]. Specifically, it has been clarified that Trypsin has its maximum affinity to cleave PAR-2 (although it can also cleave PAR-1 and 4) whereas Chymotrypsin is only able to efficiently cleave PAR-1 [84]. Importantly, it has been shown that both PAR1 and PAR2 are frequently overexpressed in many types of malignancies such as melanoma, prostate, lung, liver, colon and breast cancer [88].

Interestingly, in pancreatic cancer it has been shown that there is a strong and close relationship between PAR2-derived signaling and the TGFβ pathway [89–91]and this is in agreement with our work (described earlier) where a mixture of Trypsinogen and Chymotrypsinogen exerted potent anti-tumorigenic effects on CSCs derived from pancreatic cancer cells mainly through altering the TGFβ pathway [48]. Therefore, it is an attractive idea to consider PAR1 and PAR2 as potential targets of the anti-tumor effects of the (pro) enzyme therapy.

On the other hand, others studies have reported a pro-tumourigenic role of both PAR1 [92–94]and PAR2 [95–97], which strongly contrasts with the anti-tumor effects of proteolytic pancreatic enzymes here. However, it is necessary to note that PAR-derived signaling depends on the proteinase that binds to the receptor. Indeed, the activation or inhibition of PARs by digestive pancreatic proteases implies a complex balance between activation and inhibition of different cellular pathways. Intriguingly, Altrogge and coworkers [84] reported that Chymotrypsin could also make an inhibitory cleavage on PAR1, removing its tethered activating ligand, and making the receptor insensitive to the action of other proteinases. Additionally, it has been shown that Trypsin is able to activate PAR2-dependent signaling along with removing the tethered ligand of PAR1, thus, preventing its activation by thrombin [98]. Moreover, the complexity of PARs-derived signaling is enhanced by the fact that PARs are able to dimerize forming homodimers (two PARs of the same type) and/or heterodimers (two different PARs) in the cell surface. Such dimerization can lead to the activation of a PAR by the tethered ligand of the adjacent PAR within the dimerized structure (an event known as 'co-factoring' of PARs) which triggers a different signaling from that promoted by a single PAR [99]. Apart from the organization of PARs in the cellular membrane, it has also been reported that a single PAR can trigger alternative signaling, called 'biased' signaling depending on which proteinase cleaves the receptor N-terminal domain. For example, if PAR-1 is cleaved by thrombin the 'main or canonical' signaling is triggered, but

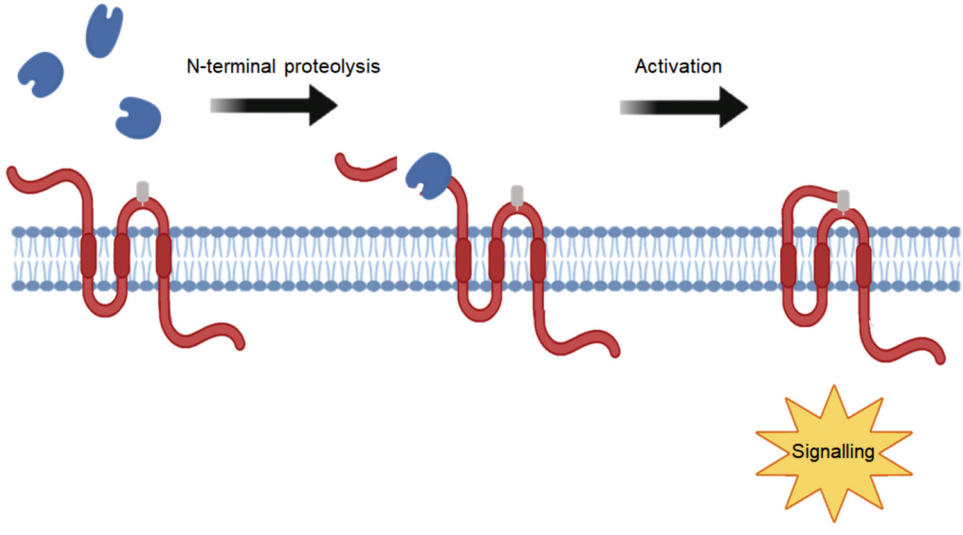

Figure 5. Activation of PAR receptors by Chymotrypsinogen/Trypsinogen.

if PAR-1 is cleaved by APC or VIIa factor, a biased signaling is prompted [100].

Finally, both PAR1 and PAR2 can transactivate other surface receptors. 'Transactivation' is defined as the activation of a second type of surface membrane receptor by the previous activation of a first one, in a mechanism which does not imply any transcription or translation processes [101]. To note, it has been pointed out that there is an importance of the transactivation of tyrosin kinase receptors by G protein-coupled receptors, like PARs, which enables G protein coupled receptors to signal even through cellular growth pathways [102]. Importantly, the transactivation of the epidermal growth factor receptor by both PAR1 [103,104] and PAR2 [105,106] has been reported. This is a very relevant fact since it has also been shown that the EGF pathway can promote the alternative splicing of RAC1 leading to the synthesis of RAC1β [107], which has been suggested to be a key factor responsible for the anti-tumor properties of pancreatic (pro)enzymes [48].

6. Conclusion

The use of a mixture of pancreatic (pro)enzymes as adjuvant therapy in cancer has been proved to be a successful strategy for reducing tumor size and improving the survival rate. Proteolytic administration also decreases inflammation and promotes activation of the immune system, both phenomena being closely linked to cancer.

The study of the structure and the structural variants of Trypsinogen and Chymotrypsinogen can give answers to their mode of action against tumor cells. The dynamics of their active sites and the prevalence of the cationic isoform in acidic environments have implications on the behavior of the (pro) enzymes in the tumor surrounding. Finally, the effect of Trypsinogen and Chymotrypsinogen on cancer cells that induces the shifting between EMT to MET, could be driven by activation of PAR1 and PAR2 receptors.

7. Expert opinion

Cancer is a question of heterogeneity: inter-patient heterogeneity; tumor-tissue specific heterogeneity, primary and metastatic tumors heterogeneity and even intra-tumor cell heterogeneity. Differences between the characteristics of the tumors developed in each patient are making it very difficult to find a definitive therapy. Current therapies rely on assaulting tumor cells by radiation or chemical agents, but these weapons also reach healthy cells causing many side effects. Moreover, the population of Cancer Stem Cells (CSCs) within the tumor mass has been described to cause metastasis and tumor relapse. The strength of these particular cells is that they do not replicate, so conventional therapies that only affect growing cells, do not harm CSCs.

The peculiar nature of cancer, its own entity within the body (preventing the immune system to respond) and the dramatic way in which it interacts with its microenvironment, is a real threat, but could also become a gateway to alternative therapies. In this respect, therapies that prevent tumor cells from maligning cells in their environment or those that wake up the immune system or decrease inflammatory effects could be effective.

For some decades now, proteolytic enzymes have been used in the fight against cancer and several beneficial clinical effects were reported. These studies demonstrated that oral enzyme therapy significantly decreased tumor-induced and therapy-induced side effects and complaints such as nausea, gastrointestinal complaints, fatigue, weight loss, and restlessness and obviously stabilized the quality of life (QoL) of cancer patients. In general, enzyme therapy was found to be well tolerated, both in adjuvant and palliative settings. Interestingly, it was also shown that pancreatic (pro)enzymes were able to stimulate the immune system response and to have anti-inflammatory potential. Furthermore, current research has demonstrated that novel pancreatic (pro)enzyme formulations decrease cell proliferation and migration, induce cell differentiation, impair angiogenesis and reduce the metastatic potential of CSCs.

The relevance of this therapeutic strategy is precisely the impact of pancreatic (pro)enzymes formulations on common characteristics of cancer such as immunosuppression, inflammatory processes induction, and surrounding tissue corruption.

We believe it is important to further study the conformational structure of pancreatic (pro)enzymes such a Trypsinogen and Chymotrypsinogen and to understand and define the specific cellular pathways that the proteases target.

Nevertheless, this new therapeutic approach would need to overcome a technical limitation regarding the supply of the (pro)enzymes. It would be necessary to establish standardized protocols for the high-scale production of the (pro)enzymes. Other alternatives, besides the use of extracts from bovine pancreas could be explored. Thus, it is key to standardize the production of a better-quality and safer pancreatic (pro) enzymes formulation, meeting the requirements of the Food & Drug Administration, and to implement clinical trials in advanced cancer patients.

Given the challenging and unprecedented times we are facing with regards to the recent global pandemic, we predict a significant and unmet need for new cancer therapies that are not only more effective and less toxic, but critically, also support the immune function of patients. As a direct result, the risk of secondary infection in people afflicted with cancer undergoing chemotherapy or radiation is considered life threatening.

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