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REVIEW ARTICLE

Use of Exogenous Enzymes in Human Therapy: Approved Drugs and Potential Applications

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Abstract: The development of safe and efficacious enzyme-based human therapies has increased greatly in the last decades, thanks to remarkable advances in the understanding of the molecular mechanisms responsible for different diseases, and the characterization of the catalytic activity of relevant exogenous enzymes that may play a remedial effect in the treatment of such pathologies. Several enzyme-based biotherapeutics have been approved by FDA (the U.S. Food and Drug Administration) and EMA (the European Medicines Agency) and many are undergoing clinical trials. Apart from enzyme replacement therapy in human genetic diseases, which is not discussed in this review, approved enzymes for human therapy find applications in several fields, from cancer therapy to thrombolysis and the treatment, e.g., of clotting disorders, cystic fibrosis, lactose intolerance and collagen-based disorders.

The majority of therapeutic enzymes are of microbial origin, the most convenient source due to fast, simple and cost-effective production and manipulation. The use of microbial recombinant enzymes has broadened prospects for human therapy but some hurdles such as high immunogenicity, protein instability, short half-life and low substrate affinity, still need to be tackled. Alternative sources of enzymes, with reduced side effects and improved activity, as well as genetic modification of the enzymes and novel delivery systems are constantly searched. Chemical modification strategies, targeted- and/or nanocarrier-mediated delivery, directed evolution and site-specific mutagenesis, fusion proteins generated by genetic manipulation are the most explored tools to reduce toxicity and improve bioavailability and cellular targeting.

This review provides a description of exogenous enzymes that are presently employed for the therapeutic management of human diseases with their current FDA/EMA-approved status, along with those already experimented at the clinical level and potential promising candidates.

Keywords: Recombinant proteins, pharmaceutical enzymes, anticancer biologics, fibrinolytic therapy, cancer enzymatic starvation, biopharmaceuticals.

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1. INTRODUCTION

The use of enzymes as therapeutic molecules has marked the beginning of a new era in the bio-pharmaceutical industry. As specific biological catalysts, enzymes make the most desirable therapeutic agents for the treatment of diseases, since they efficiently and repeatedly produce the desired effect with high specificity and affinity for their target substrates. The identification and extensive characterization of enzymes and enzymatic pathways in organisms by genomic and proteomic techniques have disclosed the large diversity of enzymatic functions and have promoted **extensive research** into their possible clinical applications. This review intends to present the use of exogenous enzymes, in their native or recombinantly generated variant forms or as fusion proteins with specific property-enhancing or targeting compounds, to catalyze novel and unprecedented biochemical reactions that have a curative effect in the human body and treat a condition of illness. Currently, enzymes are being used as excellent oncolytic, thrombolytic, mucolytic, and debriding agents. A list of the enzymes described in the review is provided in Table 1.

The enzymes have been grouped into sections based on the therapeutic applications. Enzymes employed as antineoplastic and fibrinolytic agents represent the most conspicuous group and the principal topic of this review, since cancer and cardiovascular diseases are two of the most common causes of mortality worldwide. Moreover, multiple and peculiar processes are responsible for the appearance and development of these pathologies and may be indeed specifically targeted and healed by enzyme therapeutics. Enzymes employed in replacement therapy are reviewed and discussed elsewhere in this *Current Medicinal Chemistry* issue.

2. ENZYMES USED IN CANCER THERAPY

The therapeutic activity of most conventional chemotherapeutic agents in clinical use is limited by their general cytotoxicity to proliferating cells, including some normal cells. The use of enzymes as therapeutic agents offers many advantages over conventional drugs because, due to the high specificity and affinity for their target substrates, they may operate on molecules involved in energetic, synthetic or nutritional vulnerability fundamental in the growth and survival of cancer cells. Enzymes catalyzing reactions that create a nutrient-limited environment, or able to hamper protein synthesis by acting on different stages of the processes have been widely explored and reached clinical ap-

proval for the treatment of different cancer types. Analogously, enzymes degrading metabolites, drugs or matrices components find application as co-adjuvants to implement the antitumor therapy (Fig. 1).

2.1. Enzyme-mediated Nutrient Starvation of Cancer Cells

Early in the 20th century, it was recognized that cancer cells rewire their metabolism to support the energetic and biosynthetic demands of their high proliferation rates. Mutations or changes of expression in metabolic genes can alter metabolic pathways and thus create addictions to extracellular nutrients, but even when there are no metabolic changes, cancer cells often show a greater demand for particular substances. Examples of metabolic targets for therapy are the enzymes of Krebs cycle, glycolysis, beta-oxidation of fatty acids, amino acids, and mitochondrial metabolism. Thus, enzymatic starvation is emerging as a promising strategy for the development of novel therapeutics against cancer [1]. In particular, there has **been growing** attention to glucose metabolism, which has been recognized as one of the hallmarks of cancer and to the group of enzyme drugs targeting amino acid addictions, whose efficacy has already been shown to be a powerful tool in several cases [2]. Indeed, in the field of amino acid addictions, pre-clinical and clinical work shows that some enzymes, namely L-asparaginase, L-arginine deiminase, L-methionine γ -lyase, and cyst(e)inase can be used in order to successfully remove amino acids and lead cancer cells to death [3, 4]. As human enzymes do not have the pharmacological or catalytic requirements essential for the desired therapeutic purpose, heterologous enzymes, and/or recombinant and/or engineered human enzymes are used in both animal and clinical studies. Presently, the most used are enzymes of bacterial origin. In this section, enzymes degrading amino acids and glucose are reviewed.

2.1.1. Asparagine Depleting Enzymes

Asparagine (L-Asn) is a nonessential amino acid that has a relevant role in the synthesis of a large number of proteins, in the biosynthesis of glycoproteins and in the metabolism of ammonia. Since the early 1950s, it has been highlighted that certain types of tumors, such as leukemic cells, cannot synthesize Asn because they lack or have **a deficient** expression of the enzyme L-asparagine synthetase and, consequently, are strictly dependent on Asn circulating in the bloodstream. L-asparaginase (EC 3.5.1.1, ASNase) is recog-

Table 1. Exogenous therapeutic enzymes listed in this review.

Application	Enzyme(EC)	Function/Mechanism	Source/Expression System	Approved by FDA/E MA
Cancer	Asparaginase(3.5.1.1)	Asparaginadepletion	<i>Escherichia coli</i>	1978* (FDA)2016 (EMA)
			<i>Erwinia chrysanthemi</i>	2011(FDA)
	Arginine deiminase			
	(3.5.3.6)			
Cancer	L-Arginase(3.5.3.1)	Argininedepletion	Multiple sources	
	L-Arginine			
	Decarboxylase			
	(4.1.1.19)			
Cancer	Methionine γ -lyase (4.4.1.11)	Methioninedepletion	Multiple sources	
Cancer	Cystathionine- γ -lyase (4.4.1.1)	Cysteinedepletion	Human	
Cancer	Glucoseoxidase (1.1.3.4)	Glucose deprivation	Multiple sources	
Cancer	Immunotoxins: bacterial toxins (2.4.2.36) plant ribosome inactivating proteins (3.2. 2.22) mammalian toxins	Eukaryotic protein synthesisinhi- bition	Multiple sources	1999 (FDA)
	(e.g.: RNases -3.1.27.5)			
Cancer	Hyaluronidase(3.2.1.35)	Hyaluronandegradation	Human in Chinese hamster ovarycells	2005 (FDA)
Cancer (tumorlysis syndrome)	Urate oxidase(1.7.3.3)	Hyperuricemiaprevention and treatment	<i>Aspergillus flavus</i> in <i>Saccharo- mycescerevisiae</i>	2002 (FDA)/2001(EMA)
Methotrexate(MTX) intoxication	Glucarpidase(3.4.17.1)	MTX degradationwith in anticancer therapy	<i>Pseudomonasspp.</i>	2012 (FDA)
Thrombolysis	Streptokinase(3.4.99.22) uPA (urokinase- type plasmino- genactivator) (3.4.21.73) tPA (tissue-typeplasminogen activators (3.4.21.68) alteplase tenecteplase reteplase Desmoteplase (3.4.21.68) Staphylokinase(3.4.99.22 transferred to3.4.24.29)	Plasminogenactivation	<i>Streptococcus</i> haemolyticus Human urine Human neonatal kidney cells Human melanoma in Chinese hamster ovarycells expressed in <i>E.coli</i> <i>DesmodusRotundus</i> <i>Staphylococcus</i> ausureus	1982 (FDA) 1978 (FDA) 1987 (FDA)/ 2002 (EMA) ** 2000 (FDA)/ 2001 (EMA) 1996 (FDA/EMA)
Direct-acting	Plasmin (3.4.21.7)	Fibrin degradation		
thrombolyticagents	(miniplasmin,microplasmin, deltaplasmin) Nattokinase(3.4.21.62)		<i>Bacillus subtilis</i>	
Phenylketonuria	Phenylalanine ammonia lyase (4.3.1.24)	Phenylalaninedegradation	<i>Anaboenavariabilis</i>	2018 (FDA,EMA)
Cystic fibrosis	Dornase alfa - human deoxyri- bonuclease I(3.1.21.1)	Extracellular DNA degradation	Chinese hamster ovarycells	1993 (FDA) 1994 (EMA)
Lactose intolerance	Lactase (3.2.1.10)	Lactose hydrolysis (β -galactosi- dase)	Fungi, yeasts	GRAS(FDA)
Disc herniation(chemonucleol ysis)	Chimopapain (3.4.22.6) Condoliase(4.2.2.20)	Degradation of proteoglycans in nucleus polposus Degradation of glycosaminoglyc ans (chondroitin sulfate ABC endolyase)	<i>Carica papaya</i> <i>Proteusvulgaris</i>	1982 (FDA)
Dupuytren'scontracture Peyronie'sdisease	Collagenase(3.4.24.3)	Hydrolysis ofcollagen	<i>Clostridiumhistolyticum</i>	2010 (FDA) 2013 (FDA)/ 2014 (EMA)

* The text reports the dates of approval for the PEGylated forms.

** The dates reported refer to alteplase approval for AMI treatment.

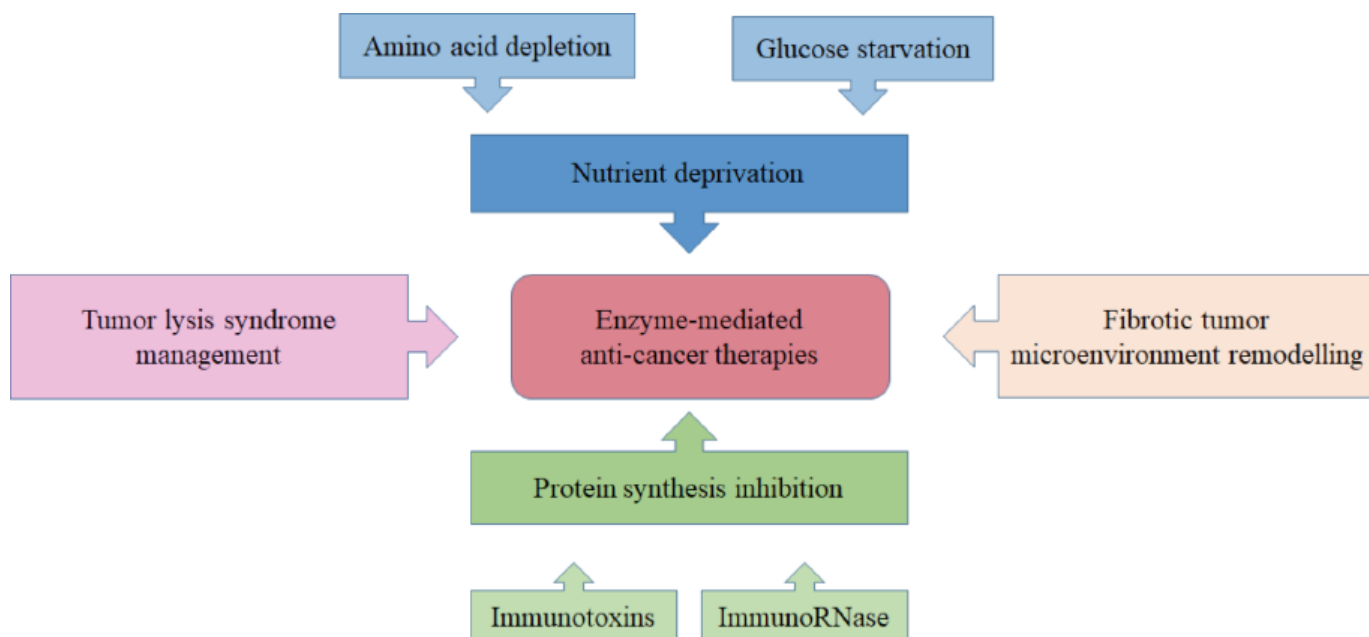
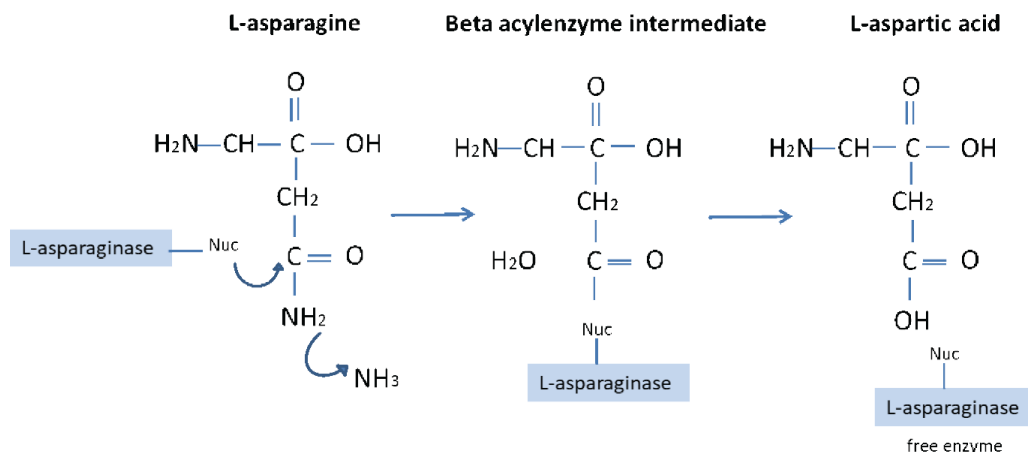


Fig. (1). Use of enzymes in anti-cancer therapies. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

nized as a very effective anticancer drug as it exploits this specific requirement of malignant cells. The application of ASNase as a chemotherapeutic agent is based on its catalytic activity of hydrolysis of L-asparagine into L-aspartic acid and ammonia. The reaction proceeds in two steps via a beta-acyl-enzyme intermediate (Scheme 1) [5]. With lower efficiency, ASNases are also capable of hydrolyzing L-glutamine to L-glutamate and ammonia.

ASNase is a relatively widespread enzyme found in many organisms (animals, plants, and microorganisms). Nevertheless, only bacteria produce a sufficient amount of enzyme to be of commercial significance. Bacteria can express two isozymes of ASNase, namely type I and type II. While both type I and type II AS-

Nases are characterized by enzymatic activity on both L-asparagine and L-glutamine, they differ for localization (extra- or intracellular), substrate affinity and quaternary structure and only type II ASNases have anti-cancer activity [6]. Bacterial type II ASNases are periplasmic enzymes, active as homotetramers, with the active site positioned in between two adjacent monomers, with a low K_m (10^{-5} M) for L-Asn and a broad substrate specificity [7]. ASNases derived from *Escherichia coli* (E-coli-ASNase) and *Erwinia chrysanthemi* (Echry-ASNase) have been shown to have the best anticancer capabilities and are presently used in clinics. The potential use of ASNase in cancer treatment dates back to 1953 when Kidd discovered that the use of guinea pig serum has a cytotoxic effect on lym-



Scheme 1. The degradation of L-asparagine catalysed by ASNase.

phoma transplanted mice [8]. Broome subsequently demonstrated that ASNase is responsible for the anti-lymphoma effect of guinea pig serum [9]. This discovery led to ASNase treatment in patients with ALL (Acute Lymphoblastic Leukemia) and Loeb *et al.* [10] stated that ASNase can be employed at any stage of ALL to obtain rapid remissions. Since then several studies including large and controlled clinical trials [11-15] demonstrated the cytotoxicity of the enzyme ASNase, alone or in combination with chemotherapy drugs, against leukemia cells and a number of other tumor cell lines that require an exogenous supply of L-ASN. In 1978, the U.S. FDA approved the use of the enzyme for the treatment of ALL and to date ASNase in combination with vincristine and steroids is routinely used in clinics around the world, with a rate of 80-90% complete remission [16-19]. The results obtained with ASNase in the treatment of pediatric ALL have led to testing the effectiveness of the drug on different tumors: acute myeloblastic leukemia, Hodgkin and non-Hodgkin lymphomas, myelosarcoma, myeloma, the highly aggressive extranodal NK/T cell nasal type lymphoma [20-22], pancreatic carcinoma [23], ovarian cancer [24, 25]. The bacterial origin often leads to immunogenicity and instability of the protein in humans causing adverse reactions during treatment [26]. In order to diminish immunological responses, a polyethylene glycol (PEG)-conjugated version of the ASNase was developed and used in clinical trials for the first time in the 1980s. This gave reduced immunogenicity, increased circulation time in the bloodstream, and higher stability at elevated temperatures [27]. Moreover, modified ASNase from *E. chrysanthemi* showed higher cytotoxicity against leukemia cells, probably due to the extended circulation of the enzyme [28]. In addition to chemical PEGylation, a less immunogenic and more stable formulation of ASNase has been obtained by loading red blood cells with the enzyme (GRASPA) [29]. More recently nano-complex delivery systems such as poly (lactic-co-glycolic) acid nanospheres [30] and lipid nanovesicles modified with chitosan have been developed [31]. However, further studies are required to determine the true potential of these methods. To overcome the immunogenicity problems of the enzyme, the best solution would be to use human ASNase. Several engineering studies have been conducted to produce human asparagine-degrading enzymes [32-34] but there are several issues regarding their clinical application. In order to improve thermal stability and immunogenic response of the enzyme, numerous studies are underway to identify and characterize ASNases with antineoplastic activity from other

prokaryotic and eukaryotic sources [35]. Recently, in order to enhance its bioactivity, ASNase was loaded onto gold nanoparticles along with a cell-penetrating peptide Arg-Gly-Asp (RGD). L-asparaginase activity was significantly increased and the presence of RGD peptide could direct the enzyme to target cancer cells and improve anticancer efficiency [36]. To date, there are 3 different types of ASNase available to treat ALL: (i) native *E. coli* ASNase (Elspar, Kidrolase, Leunase and Spectrila); (ii) PEGasparaginase/PEGaspargase, a pegylated version of ASNase derived from *E. coli* (On-caspar) that was approved by both the FDA (2006) and EMA (2016); Calaspargase pegol (SC-PEG), a more stable PEGylated ASNase molecule approved by FDA (2018) and, (iii) an *E. chrysanthemi* based native ASNase (Erwinase,) approved by FDA (2011).

2.1.2. Arginine Depleting Enzymes

Arginine is a key molecule serving as a precursor for the synthesis not only of proteins but also of nitric oxide, urea, polyamines, proline, glutamate, creatine and agmatine. Owing to the involvement of arginine in so many cellular pathways, arginine dependence on tumor cells has rapidly emerged as a potential target for cancer. Arginine is a semi-essential amino acid that can be obtained from daily food intake or synthesized in the body through the urea cycle using L-citrulline as a substrate. Synthesis of arginine is catalyzed by argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) or by the activation of a *salvage* pathway in which arginine is synthesized by ornithine transcarbamylase (OTC). Several tumors have downregulated arginine metabolizing enzymes and depend on exogenous arginine for their survival [37]. The most represented arginine-auxotrophic human cancers are metastatic melanoma, Hodgkin's and non-Hodgkin's lymphoma, osteosarcoma and promyelocytic leukemia, hepatocellular carcinoma, breast carcinoma [38-40] and several tumors derived from epithelial tissues [41]. Three L-Arg depleting enzymes have been extensively investigated for their cytotoxic activity: arginine deiminase (EC 3.5.3.6, ADI), L-Arginase (EC 3.5.3.1, ARGase), and L-Arginine decarboxylase (EC 4.1.1.19, ADC). The bacterial arginine deiminase is the most promising of the arginine-degrading enzymes. ADI catalyzes the irreversible conversion of L-arginine to citrulline and an ammonium ion and is highly specific for arginine. ADI has been obtained from several microbial sources but the most extensively studied is the one derived from *Mycoplasma arginini* (Ma-ADI) [42]. It is a homodimer of 90 kDa with two distinct conformations, an open form (apo-enzyme) and a closed form

(enzyme-substrate complex). Ma- ADI anti-cancer activity firstly was reported by Miyazaki *et al.* [43] for infected human tumor cells and subsequently was observed in several cancer cell lines deficient for argininosuccinate synthetase [44]. *In vivo* studies pointed out that the native ADI is unstable, immunogenic and is eliminated rapidly from the circulation. In order to reduce the antigenicity and prolong half-life in serum, ADI was conjugated with 20 kDa PEG (ADI-PEG 20; pegargininase) [45]. ADI-PEG20 has completed phase III trials in patients with advanced hepatocellular carcinoma, is currently undergoing phase II studies in patients with acute myeloid leukaemia, malignant melanoma, non- Hodgkin's lymphoma, small cell lung cancer, soft tissue sarcoma, mesothelioma, and phase I studies in patients with glioblastoma, non-small cell lung cancer, pancreatic cancer, solid tumors, uveal melanoma [46, 47]. Numerous studies to improve the properties of the enzyme (optimal pH, K_m , stability and solubility) are documented [42, 48].

ARGase is a mammalian enzyme, which catalyzes the conversion of arginine to ornithine and urea. The enzyme has been tested on a variety of cancer cell lines and in animal models [47, 49, 50] and has been considered as a potential drug candidate over a period of six decades. Unfortunately, low substrate specificity (high K_m of 2–4 mM), short plasma life, optimum alkaline pH (pH 9.3) and the requirement of Mn^{2+} for activity limit *in vivo* its applications. To counteract these limitations, various protein-engineering approaches have been employed [48, 51, 52]. PEGylated recombinant human ARGase I (BCT-100) exhibits increased stability and improved catalytic properties. It has been tested in several *in vitro* and *in vivo* models showing efficacy in reducing cancer cells growth mainly in leukemia and hepatocellular carcinoma models and is undergoing Phase II clinical trials [53, 54]. Recently, it has been reported that Ni^{2+} and Co^{2+} -substituted ARGase variants display higher catalytic activity than wild type and inhibit at least eight types of cancer cell lines [55]. ADC, which metabolizes arginine to agmatine, has limited anticancer potential due to the toxicity of the reaction product for normal cells and requires further development for a possible therapeutic use [44].

2.1.3. Enzymes Acting on Sulfur Metabolism

Sulfur-containing compounds are involved in a series of reactions that produce energy and biomass necessary for tumor growth [56]. Cysteine and methionine not only provide the sulfur necessary to support redox chemistry, but their metabolism produces intermediate

metabolites that compose the cellular antioxidant system, regulate important signaling pathways and mediate the epigenetic control of gene expression, which are all involved in cancer proliferation. Enzymes degrading sulfur-containing amino acids have thus great potential as antiproliferative agents and are currently under investigation.

2.1.3.1. Methionine γ -lyase

Methionine is an essential amino acid playing different and crucial roles in mammalian metabolism and cell proliferation such as protein synthesis, glutathione and polyamine synthesis, and methylation of DNA [57]. Methionine metabolism has been connected to cancer at several levels and indeed numerous studies have revealed that while normal cells do not suffer from methionine deficiency since they possess the ability to grow on homocysteine, one of methionine metabolic precursor, in the same conditions, most cancer cells cannot proliferate [58]. Methionine dependence is a general metabolic defect of cancer and has been attributed to a combination of molecular events, including deletions, polymorphisms or alterations in the expression of genes in the methionine *de novo* and salvage pathways [58, 59]. These observations have highlighted methionine depletion as a tumor-specific therapeutic strategy. A straightforward way to achieve a reduction of L-Met levels, apart from a methionine-restricted diet that is limited due to the consequent rapid weight loss and other toxic effects, is the utilization of a methionine-degrading enzyme, L-methionine γ -lyase (EC 4.4.1.11, MGL). MGL is a pyridoxal 5'-phosphate (PLP) dependent enzyme that catalyzes cleavage of methionine into methanethiol, α -ketobutyrate and ammonia. MGLs from bacteria are the best characterized and studied form of the enzyme [60]. Recently, the Morozova group provided evidence for the cytotoxicity of MGL from *Clostridium sporogenes*, *Clostridium tetani*, and *Citrobacter freundii* using K562, PC-3, LnCap, MCF7, SKOV-3, and L5178y tumor cell lines [61]. *Pseudomonas putida* MGL is the most extensively investigated with regard to its *in vitro* and *in vivo* anti-cancer activity [57, 62]. Bacterial MGLs are generally cytosolic homotetramers, with two tightly associated dimers forming the active site at the dimer interface. Upon treatment with MGL, cancer cells are reported to arrest in the S/G2 phase of the cell cycle, to be susceptible to spontaneous death and become hypersensitive to chemotherapeutic agents [63]. Lately, it was reported that even short-term starvation of methionine, but not other amino acids, leads to a dramatic interruption of the onset of the tumor, which is mostly attributable to a block of cellular methylation

in tumor-initiating cells (TICs) [64]. *In vivo* experiments proved the efficacy of MGL, alone or in combination with other chemotherapeutics like vincristine, 5-fluorouracil, and cisplatin, as a molecule with great potential for cancer therapy of numerous tumors (i.e. glioblastoma, neuroblastoma, melanoma, colon cancer, lung cancer, brain cancer and prostate cancer) [59, 65-68]. With the aim to improve the therapeutic properties of the enzyme, MGL from *C. freundii* was engineered by site-saturation mutagenesis. The authors identified important residues coupled to active site dynamics that contribute to activity [69]. In addition to mediate methionine-dependent cancer cells death, MGL is also used as a specific activator of the pro-drug selenomethionine resulting in the destruction of tumors following the formation of toxic superoxide by thiol oxidation [70, 71]. Despite numerous reports showing the effectiveness of the protein, the demonstrations of antitumor efficacy of MGL in clinical studies are preliminary and limited in number and the enzyme has not yet been approved by the FDA. The drug presents some limitations mainly due to its instability in the bloodstream and to its immunogenicity. Several strategies have been adopted to improve bioavailability and delivery of MGL to cancer cells. These include free and PEG-conjugated MGL [72, 73], stealth PLGA/liposome-containing MGL [74], silica gel-entrapped MGL [75] and gene therapy-based on adenoviral vector carrying the MGL gene [76]. To reduce the immunogenicity of MGL, a methionine-degrading enzyme was created *de novo* by reengineering the structurally homologous PLP-dependent human enzyme cystathionine- γ -lyase to change its substrate specificity and catalyze methionine degradation [67, 77]. In addition, *in vivo* studies on erythrocytes loaded with MGL clearly demonstrated that encapsulation of MGL in erythrocytes not only strongly improved the enzyme half-life but also provided an active cofactor [78]. One important limitation in the use of the enzyme is indeed the requirement for PLP as a cofactor for its catalytic activity. Since PLP availability in the bloodstream is limited because of its prompt assimilation and degradation, concurrent loading of MGL and pyridoxine into erythrocytes, that can convert pyridoxine to PLP, can solve this limitation.

2.1.3.2. Cyst(e)inase

Cysteine is a semi-essential sulfur-containing amino acid mostly acquired from the extracellular environment through the xCT transporter as cystine. Otherwise, it can be synthesized from methionine and serine through the trans-sulfuration pathway. Although cysteine is the least abundant amino acid in the cell, it is recognized as one of the most important for cell sur-

vival and growth. It is widely used throughout the cell as a nutrient, stimulates cellular bioenergetics as a substrate for the production of hydrogen sulphide (H₂S) and is critical for protein folding. Cysteine is a mediator for the oxidative stress response as a component of glutathione (GSH), an important intracellular antioxidant broadly implicated in tumor initiation, progression, metastasis, and resistance [79]. Since some tumors have a significantly lower expression of transsulfuration enzymes, due to transcriptional silencing, the import of extracellular L-Cys becomes crucial. Further, an iron-dependent, non-apoptotic form of cell death (ferroptosis) has recently been described as the result of cysteine insufficiency leading to a collapse of intracellular glutathione level, an accumulation of reactive oxygen species and excessive peroxidation of membrane phospholipids [80]. Having such a wide relevance in cancer, alteration of cysteine homeostasis appears to be a promising strategy to kill cancer cells. A way for decreasing the intracellular cysteine level is pharmacological depletion by cystathionine- γ -lyase (CGL, EC 4.4.1.1). Tetrameric CGL is a PLP-dependent enzyme that catalyzes the conversion of L-cystathionine into α -ketobutyrate, L-Cys and NH₄⁺. CGL can also accept cysteine and cystine as substrates but its kinetic rates are too slow for clinical applications [81]. Cyst(e)inase, an engineered and pharmacologically optimized CGL, strongly reduces cysteine and cystine pools. Treatment with cyst(e)inase selectively causes cell cycle arrest and death in cancer cells due to depletion of intracellular GSH, ensuing elevated reactive oxygen species (ROS) and consequent ferroptosis. It was observed that the engineered enzyme is well-tolerated in mammals for prolonged periods, has a long half-life, and determines cancer cell cytotoxicity in multiple murine tumor models [81-83]. Administration of cyst(e)inase in combination with auranofin resulted in the sensitization of cyst(e)inase-resistant tumors with a favorable safety profile [84]. In addition to these studies on solid tumors, recently, it has been reported that the treatment with the engineered enzyme leads to selective suppression of human leukemia stem cells, with no detectable effects on normal hematopoietic cells [85]. All the obtained results indicate that cyst(e)inase may be a useful therapeutic strategy and justify further clinical evaluation.

2.1.4. Glucose Depriving Enzymes

2.1.4.1. Glucose Oxidase

In contrast to slowly proliferating normal tissues, most cancer cells show a markedly increased glucose consumption, producing energy through anaerobic gly-

colysis (Warburg effect) rather than *via* respiratory metabolism, even in the presence of normal O₂ levels. As a result, cancer cells must burn almost 15-fold the amount of glucose to get the same amount of energy compared to normal cells. In addition to amino acid starvation, glucose depletion has been extensively studied for cancer treatment. Glucose oxidase (GOx, EC 1.1.3.4), a homodimer, catalyzes the oxidation of glucose into gluconic acid and hydrogen peroxide in the presence of oxygen. This process can directly consume glucose, **block** the energy nutrition source of tumor cells and **inhibit** their proliferation. The activity of GOx also causes: 1) oxygen consumption, which increases tumor hypoxia; 2) generation of gluconic acid, which enhances the acidity of the tumor microenvironment; 3) production of H₂O₂, which raises tumor oxidative stress levels [86]. Therefore, in addition to its use in cancer diagnosis as GOx-based biosensor [87], glucose oxidase emerges as a promising anticancer drug [88]. **Besides** cancer starvation therapy, in recent years, GOx catalysis has been further integrated with other therapeutic methods (radiotherapy, chemotherapy, hypoxia-activated therapy, oxidation therapy, photodynamic therapy, and/or photothermal therapy) to achieve enhanced synergistic therapeutic effects [89]. Although GOx has good potentiality in tumor therapy, some characteristics, such as inactivation, immunogenicity, instability after exposure to biological conditions, short *in vivo* half-life (~30 min at 37 °C), systemic toxicity, poor delivery efficiency, and high cost for the preparation and purification, limit its biomedical application [90]. In recent years, a variety of nanocarriers including magnetic nanoparticles, mesoporous silica nanoparticles, metal-organic structures and polymers have been investigated with the aim to protect the enzyme from the surrounding environment and to preserve its activity [91-93]. However, these nanocarriers are usually undegradable and may cause biotoxicity. Recently, a degradable nanocarrier for precise GOx-based starvation has been developed as an effective anticarcinogen for clinical applications [94].

2.2. Enzymes Targeting High Protein Synthesis in Cancer: Immunotoxins

Immunotoxins (ITs) are an anticancer therapeutic option based on chimeric proteins that combine a cancer-targeting moiety with the catalytic part of a toxin, responsible for toxin-induced lethality. This simple and flexible conceptual design allows multiple combinations of these two major components with unlimited prospects to generate potential therapeutic agents with targeted selectivity [95-98]. The appropriate surface

markers - unique or highly expressed in **tumors** but absent or with relatively restricted expression in healthy tissues - serve to deliver the toxin to the designated tumor. Indeed, antibodies, antibody fragments, hormones, lectins, cell-penetrating peptides, growth factors, and cytokines have been tested to dictate a new binding specificity. Analogously, the toxin payloads carried by ITs may derive from any source such as bacteria (e.g., *Corynebacterium diphtheriae* diphtheria toxin [DT], *Pseudomonas aeruginosa* exotoxin A [PEA]), plants (e.g., ribosome-inactivating proteins (RIPs) such as ricin, mistletoe lectin-1, saporin, gelonin and bouganin), or mammals (e.g., ribonucleases [RNases]) and act as an extremely toxic effectormoiety [99]. Bacterial toxins (EC 2.4.2.36) catalyze the ADP-ribosylation of the mammalian elongation factor eEF2, leading to its inactivation and blockage of the aa-tRNA in the A-site of the ribosome, causing irreversible protein synthesis arrest and subsequent cell death. Bacteria-derived toxins generally comprise a cell-binding domain for cellular internalization, a translocation domain and motif for escape from subcellular compartments into the cytosol, and a catalytic domain to exert their toxic effects. Among them, PEA and DT have been used most extensively. DT is a 535- amino acid single-chain Y-shaped protein formed by the N-terminal enzymatic A domain linked by disulfide bonds to the C-terminal binding B domain. The latter is composed of a nine α -helices transmembrane domain, which triggers a pH-dependent membrane insertion, and a heparin-binding EGF-like growth factor receptor-binding domain, which assists the passage of fragment A (*via* the translocation domain) into the cytosol. PEA is a single polypeptide chain with a size of 66 kDa, containing 613 amino acids with an N-terminal binding domain recognizing the low-density lipoprotein receptor-related protein 1 surface receptor responsible for translocation to the cytosol of the enzymatically active C-terminal fragment.

RIPs, a heterogeneous family of toxins, act as N-glycosidases (EC 3.2. 2.22) and irreversibly inhibit eukaryotic protein synthesis by inactivating the ribosome *via* depurination of ribosomal RNA [100]. RIPs have been classified into two subgroups: type-1 toxins are monomeric proteins of approximately 30 kDa with only a catalytic domain, while type-2 holotoxins are heterodimeric proteins that contain an enzymatic A-chain of approximately 30 kDa linked *via* a disulphide bond to a lectin-like B-chain of approximately 35 kDa, which is able to bind to sugar-containing receptors on the cell membrane and facilitate internalization. Toxicity greatly varies among

RIPs with some being lethal at subpicomolar concentrations, but type-2 are generally more potent than type-1 RIPs. ITs have historically evolved into four generations. At first, whole antibodies chemically conjugated to native toxins were affected by non-specific toxicity due to the affinity of the native toxin B-chain for normal cells. Deglycosylated variants of ricin or chemically 'blocked ricin' on galactose-binding sites by affinity ligands [101], were produced to impede carbohydrate-mediated off-target interactions and increase circulating half-life. The addition of PEG was also attempted to advance ITs pharmacological properties and mitigate immunogenicity [102]. In second-generation ITs, removal of cell-binding domains generated toxin fragments that were covalently joined to the antibody by chemical conjugation. Even if off-target toxicity was reduced, several issues such as limited production, poor stability, large molecular size that affected penetration capability, heterogeneous composition due to random conjugation and immunologic side effects due to the non-human origin of components persisted and limited their usefulness. Benefiting from advances in recombinant DNA technology, third-generation ITs were genetically engineered as fusion proteins of toxin and carrier moiety that grant higher control over ITs design and production. In addition, toxins have been genetically modified to improve activity, stability and/or to decrease immunogenicity. Truncated DT389 (comprising amino acids 1 to 389) and PE40, PE38 and PE24 (according to residual molecular weight) produced by deletion of unnecessary domains are the most frequently used versions in recombinant ITs construction. In addition, epitope-depleted mutants were created by removal of the immunogenic B- and T-cell epitopes-rich regions and by point mutations at T- and B-cell epitopes within the toxin catalytic domain in PE24, gelonin and bouganin [103, 104]. The V6A substitution was introduced by site-directed mutagenesis into the DT motif to prevent vascular leak syndrome, one of the main side effects of ITs [105]. Shortened versions of the antibody part such as single-chain variable fragment, disulfide-stabilized variable fragment, nanobodies, or combination of bispecific antibodies for simultaneous dual-antigen targeting, were tested in order to decrease immunogenicity, to reduce conjugates size to facilitate tumor penetration, to enhance potency and decrease off-tumor toxicity. Different expression systems such as bacteria, yeast, Chinese hamster ovary cells, or insect cells were employed to obtain a more homogeneous and cost-effective production [106]. DT-resistant *Pichia pastoris*, and *C. diphtheria* were tested as secretion expression systems obtaining the fully-folded biologically

active, monomeric form of DT into the culture medium and reducing previously experienced aggregation problems.

Currently, three toxin-mediated therapeutics have been approved by FDA. Denileukin Diftitox (Ontak®, Eisai Inc.), was the first immunotoxin approved in 1999 for the treatment of persistent or recurrent cutaneous T-cell lymphoma, where DT389 was fused to recombinant full-length human interleukin-2, in order to target the highly expressed IL-2 receptor on malignant T-cells [107]. Despite its clinical effectiveness, in 2014, it was clinically discontinued due to developability and production limitations. In 2018, FDA approved Tagraxofusp (Elzonris®, Stemline Therapeutics), a CD123-directed cytotoxin composed of the catalytic and translocation domains of DT fused to a full-length human IL-3, as the first therapy for blastic plasmacytoid dendritic cell neoplasm in adults and in pediatric patients 2 years and older [108]. In 2020, EMA granted its marketing authorization. Analogously, Moxetumomab pasudotox (Lumoxiti®, Astra-Zeneca), consisting of the binding fragment of an anti-CD22 antibody fused to PE38, received approval in the same years by FDA and EMA for the treatment of adults with relapsed or refractory hairy cell leukemia who received at least two prior systemic therapies, including treatment with a purine nucleoside analog [109]. At present, no ITs are currently approved for solid tumors, since approved ITs products are restricted to the treatment of hematological cancers due to vascular accessibility and the presence of clusters of differentiation on the cell surface. Numerous groups are working on ITs inherent problems and a multitude of novel constructs assessing the appropriate combination of tumoricidal toxin and targeting components with high specificity and internalization efficiency are continuously being explored. Nanotechnology-based delivery systems and platforms for combination therapies have also been under investigation [110, 111]. Although numerous ITs were successful at the preclinical level and some reached clinical trials, clinical development of ITs is still hampered by the short plasma half-life, the narrow therapeutic window mainly due to the off-target toxicities, and immunogenicity [112].

The most recent fourth-generation ITs use human or humanized antibody fragments and/or human endogenous cytotoxic enzymes such as RNases to eliminate immune reactions. Human RNases possess limited intrinsic cytotoxicity; however, they can acquire cytotoxic properties once linked to cancer-specific antibodies or targeting components in a chimeric construct referred to as immunoRNase [113].

Alternatively, several RNases derived from different sources such as bacteria (binase), fungi (streptomyces ribonuclease), mammals (bovine seminal ribonuclease) and amphibians (ranpirinase and amphinase) proved to be inherently cytotoxic against cancer cells and have been tested as either stand-alone anticancers or targeted immunofusions [114-116]. Cytotoxic RNases (EC 3.1.27.5), are basic, single-chain proteins that have ribonucleolytic activity directed predominantly on tRNA, resulting in the inhibition of protein synthesis. The RNA damaging action is exerted also on noncoding RNAs, especially miRNA, with multiple effects on the regulation of gene expression [117].

ImmunoRNases are currently restricted to testing at the preclinical level, with the exception of ranpirinase, whose investigation advanced to clinical trials [118]. Ranpirinase (or oncornase), isolated from oocytes of *Rana pipiens*, consists of 104 amino acids, has a molecular mass of 11.8 kDa and shows typical fold of the pancreatic-type RNases. The highly compact conformation, characterized by disulfide bonds and a pyroglutamyl residue at the N-terminus, make the protein resistant to proteases and heat-inactivation. The compact structure and the lack of key loop residues involved in the interaction with the RNase inhibitor impede the formation of a tight complex. Thus, its ability to evade the cytosolic RNase inhibitor together with its very high conformational stability and its protease resistance are important determinants for its toxicity [119]. Mechanisms of ranpirinase tumor selectivity remain elusive. The strongly cationic character of ranpirinase is likely to promote the interaction with the highly anionic tumor cells membranes due to a greater level of sialic acid-rich gangliosides and altered phospholipid content. Cancer cells penetration of ranpirinase is thought to occur by energy-dependent endocytosis. It is likely that different and more efficient intracellular routing in tumor cells, and greater susceptibility to RNA degradation of fast-growing tumor cells may also account for tumor selectivity of ranpirinase [117].

Ranpirinase repurposing as an antiviral drug for broad-spectrum activity against double-stranded DNA viruses [120], retroviruses [121, 122], and single-stranded RNA viruses including Ebola virus [123] is currently under investigation.

2.3. Hyaluronan-degrading Enzymes Remodeling Tumor Microenvironment

Abnormal fibrotic regions consisting of a dense extracellular matrix immersed in a viscous interstitial fluid characterize many tumors [124]. Among the ma-

jor structural components is hyaluronan (HA), a large, hygroscopic, unbranched, hydrophilic glycosaminoglycan polymer, whose excessive production causes the extracellular matrix to swell, resulting in high tumor interstitial pressure, tumor vasculature collapse, and tumor hypoxia [125]. The correlation between an abundant fibrotic tumor microenvironment and more aggressive tumor phenotype and poor prognosis is demonstrated [126, 127] and likely related to HA-generated mechanical barrier that shields cancer cells from immune cell attack and prevents the anticancer agents' penetration contributing to tumor resistance to antineoplastic agents [128]. However, it may also be linked to HA's direct involvement in signaling processes, including cell proliferation, migration, adhesion, invasion [129]. Thus, hyaluronidase, an endoglycosidase (EC 3.2.1.35) that degrades HA by hydrolysis of 1,4-beta-D-glycosidic linkages between N-acetyl-beta-D-glucosamine and D-glucuronate residues, acting as an antifibrotic agent, may help in remodeling and restoring the functionality of the tumor stroma and re-establishing the penetration of cancer therapeutics [130]. rHuPH20 (Hylenex, Halozyme) is a proprietary soluble 61 kDa-fragment of human testicular hyaluronidase. It is a glycosylated single-chain protein recombinantly produced in Chinese hamster ovary cells. Its use was approved by FDA in 2005 as an adjuvant agent to enhance dispersion and absorption of subcutaneously delivered therapies. In particular, its use as a diffusion promoter for subcutaneously administered antineoplastic drugs obtained FDA approval in 2017 in combination with rituximab (Rituxan Hycela, Genentech Inc.), a CD20-directed cytolytic monoclonal antibody for the treatment of adult patients with follicular lymphoma, diffuse large B-cell lymphoma, and chronic lymphocytic leukemia [131]; in 2019, in association with trastuzumab (Herceptin Hylecta), a potent humanized anti-HER2 receptor monoclonal antibody, for the treatment of HER2-positive breast cancer [132, 133]; in 2020, in combination with pertuzumab and trastuzumab (Phesgo, Genentech, Inc.) in the treatment of all stages of HER2-positive breast cancer in combination with chemotherapy [134]. Pertuzumab (Perjeta) is a recombinant humanized antibody directed against HER2 extracellular domain II required for the heterodimerization of HER2 with other HER receptors. Analogously, in 2020, its combination with daratumumab (Darzalex Faspro), a CD38-directed cytolytic antibody, received FDA and EMA approval for the treatment of adult patients with newly diagnosed or refractory multiple myeloma [135].

A PEGylated version of rHuPH20, pegvorhyaluronidase alfa (PEGPH20), was developed for the systemic treatment of HA-high tumors [136]. Pegylation was reported to enable prolonged systemic exposure to rHuPH20. Clinical trials have examined the potential of PEGPH20 under different formulations in combination with partner chemotherapeutics in the treatment of several cancers, especially high metastatic pancreatic ductal adenocarcinoma [137-140]. However, in 2019, the Halozyme Therapeutics discontinued PEGPH20 clinical development because of disappointing clinical trial results [124].

Modification of nanocarrier surfaces through coating with hyaluronidase was applied to enhance nanocarrier diffusion into solid tumor and chemotherapeutic drugs efficacy [141]. Nanoformulated hyaluronidase in silica nanoparticles [142], in PLGA-PEG nanoparticles [143], rHuPH20-anchored to red blood cell membrane-camouflaged nanoparticles [144]; hyaluronidase-loaded albumin nanoparticles co-supplied with paclitaxel and the hyperthermal agent indocyanine green [145] and a tumor-microenvironment-responsive multistage drug delivery system nanoparticles composed of an amphiphilic pH-sensitive polymer mPEG-PDPA-PG encapsulated with epirubicin and hyaluronidase in association with matrix metalloproteinase-2-degradable gelatin and cell-penetrating peptide iRGD [146] have been described.

2.4. Enzymatic Treatment of Hyperuricemia in Cancer Cells

Tumor lysis syndrome represents a group of metabolic complications induced by chemotherapy, which causes a rapid and massive destruction of tumor cells with the release of their intracellular contents - and thus of large amounts of nucleic acids - into circulation. Purine catabolic pathway converts nucleic acids-derived purine to hypoxanthine, xanthine, and then to uric acid via the xanthine oxidase enzyme. Due to a nonsense mutation in the coding region of the gene that inactivates the urate oxidase, uric acid is the end product of purine metabolism in humans [147]. Thus, catabolism of large amounts of nucleic acids to uric acid leads to hyperuricemia. When uric acid exceeds the renal capacity of elimination, uric acid crystals precipitate in the renal tubules, leading to renal vasoconstriction, decreased renal flow, oxidation, inflammation, renal damage, acute kidney injury and potential renal failure. Since tumor lysis syndrome is a life-threatening complication of chemotherapeutic intervention, urate oxidase (EC 1.7.3.3) is widely used as a uricolytic

agent in patients with cancer for the prevention and treatment of hyperuricemia during chemotherapy [148]. This tetrameric enzyme composed of identical subunits of about 34 kDa catalyzes the oxidation of uric acid to the soluble metabolite allantoin, which is rapidly and safely excreted by the kidneys. The *Aspergillus*-derived recombinant rasburicase produced by genetically modified *Saccharomyces cerevisiae* has replaced the non-recombinant urate oxidase (Uricozyme®, Sanofi-Aventis) extracted from *Aspergillus flavus* and first introduced in therapy in France in 1975 and in Italy in 1982 [149]. The recombinant enzyme is produced in higher yields and has higher activity and purity. Rasburicase (Elitek/Fasturtec, Sanofi Aventis) is approved by the FDA and EMA for the prevention and treatment of hyperuricemia in pediatric and adult patients with solid tumors and hematologic malignancies receiving anticancer therapy. Rasburicase is contraindicated in glucose-6-phosphate dehydrogenase deficient-patients, which are at risk of hemolysis and methemoglobinemia, since erythrocytes are subject to the oxidative stress of hydrogen peroxide produced by the rasburicase-catalyzed degradation of uric acid [150]. The heterologously expressed rasburicase is potentially immunogenic, although less than the non-recombinant urate oxidase [151]. PEG-conjugated urate oxidase pegloticase (Krystexxa), a recombinant mammalian (porcine/baboon variant) enzyme produced in *E. coli* and conjugated with 10-kDa monomethoxyPEG, is less antigenic and has a longer half-life but is not currently approved for tumor lysis syndrome, while it is approved for the treatment of chronic gout in adult patients refractory to conventional therapy [152-154]. In alternative to PEGylation, PASylation - the conjugation with natively disordered biosynthetic polymers made of Pro, Ala and/or Ser - has been positively applied to urate oxidase to increase stability, solubility and half-life [155].

To prolong circulation time, reduce immunogenicity and improve the inherent poor protein stability, novel delivery vehicles of urate oxidase were recently explored. A biohybrid material responsive to uric acid pulses was employed to coordinate urate oxidase release from the matrix to serum uric acid concentrations [156]. Nanosomal microassemblies of semipermeable lipid membranes were prepared to encapsulate urate oxidase inside an alkaline environment beneficial for its catalytic reactions [157]. Efficient cascade reactions were generated by simultaneous entrapment of urate oxidase and catalase into nanosized liposomes, where catalase removed the urate oxidase-generated cytotoxic side-product hydrogen peroxide and acceler-

ated catalysis [158, 159]. Analogously, a catalase-mimicking gold nanoparticle nanozyme was studied in association with urate oxidase in a one-pot enzyme-nanozyme system [160] or using biocompatible pluronic- based nanocarriers [161] for the treatment of hyperuricemia.

3. ENZYMATIC TREATMENT OF MTX INTOXICATION WITH GLUCARPIDASE

Carboxypeptidase G (EC 3.4.17.1) is a class of enzymes able to cleave a terminal glutamate moiety from folic acid and related molecules, polyglutamate derivatives of folic acid and also from small peptides [162]. These enzymes were first identified in pseudomonads and their physiological function is related to the ability to grow on folates as the only carbon and nitrogen source [163]. Carboxypeptidase G1 was isolated from *Pseudomonas stutzeri* in 1971 [163], purified and characterized. The enzyme showed good activity with many reduced and oxidized folate derivatives, with a K_m lower than 10 μM for pteroylglutamic acid, aminopterin and methotrexate (MTX). Later, a carboxypeptidase with distinct properties but equally able to process folate and folate analogs was isolated from *Pseudomonas spp.* and named carboxypeptidase G2 [162, 164]. Carboxypeptidase G2 (CG2) is a zinc-dependent metalloprotease with periplasmatic localization and exopeptidase activity. CG2 from *Pseudomonas spp.* is a dimer and each subunit is formed by two domains: a dimerization domain and a catalytic domain that contains two zinc ions (Fig. 2). The ions are bound to the side chains of active site residues and collaborate in the formation of a bridge structure where Asp141 coordinates both ions that in turn coordinate a water molecule. This water molecule acts as the nucleophile in the attack to the amide bond [165]. The hydrolytic activity of CG2 on folate gives Glu and pteric acid, while on MTX it releases, in addition to Glu, the derivative 2,4-diamino-N10-methylpterioic acid (DAMPA) (Fig. 2). The reported favorable selectivity of action towards MTX with respect to 5-methyl tetrahydrofolate, the circulating form of folate, [164] makes this enzyme a better candidate for therapeutic applications. However, only K_m values of different carboxypeptidases towards a variety of folates, but not the k_{cat} values, are reported in the literature [163, 164, 166], thus hampering a rigorous comparison of catalytic efficiency. CG2 has at least three potential applications in therapy: i) as antineoplastic exploiting its folates- depletion activity; ii) as rescue agent for MTX intoxication in anticancer therapy; iii) in antibody-directed enzyme prodrug therapy (ADEPT) to activate

prodrugs in the tumor site [163, 164, 166, 167]. The only currently approved use of CG2, also referred to as glucarpidase, is in the treatment of MTX toxicity [168]. MTX is an antifolate developed in the second half of the 20th century as a chemotherapeutic agent and is still used today to treat a variety of cancers and autoimmune diseases [169]. Dosing issues often arise along with MTX treatment, partly due to interindividual variability in the tolerance to the drug and to the need of treating patients with high- dose MTX [170]. Doses ranging from 50 to 900 mg/m² of body surface area are able to produce toxic effects, also involving acute renal dysfunction [171]. MTX is mainly excreted through urine, thus renal impairment further exacerbates drug toxicity. In 2012, FDA approved the use of recombinant glucarpidase from *Pseudomonas sp.* (strain RS-16), under the brand name Voraxaze®, for the treatment of toxic plasma MTX concentrations [172, 173] that can arise from high-dose treatment associated with a patient's increased susceptibility or from an accidental overdose. In 2020, WEP Clinical announced its partnership with BTG Specialty Pharmaceuticals for the distribution of Voraxaze® in the UK and European markets and submission of a Marketing Authorisation Application to the EMA [174]. The drug is indicated for patients under MTX treatment who show renal impairment in association with a high plasma concentration of MTX (higher than 1 mM). The products of MTX hydrolysis by glucarpidase, i.e. Glu and DAMPA, are both non-toxic and DAMPA is readily eliminated through urine or modified in the liver before excretion [175]. The preparation contains lyophilized enzyme produced in *E. coli* in single-use vials that contain 1000 U of the enzyme (each unit corresponds to the amount of enzyme that cleaves 1 $\mu mol/L$ of methotrexate per minute at 37 °C). The enzyme is usually administered by intravenous injection of 50 U/kg body weight but can also be used intrathecally [172, 176-178]. No severe adverse effects associated with the administration of glucarpidase have been reported, the most frequent ones being flushing, vomiting, fever and headache [166]. Immunity can develop following repeated administrations [167]. The enzyme dose required for efficient treatment of MTX toxicity is relatively high. For this reason, protein engineering has been used to improve the catalytic efficiency of the enzyme, with limited but encouraging results [167].

4. ENZYMES IN CARDIOVASCULAR DISEASES: FIBRINOLYSIS

Cardiovascular disorders (CVDs), including myocardial infarction, ischemic stroke, pulmonary embo-

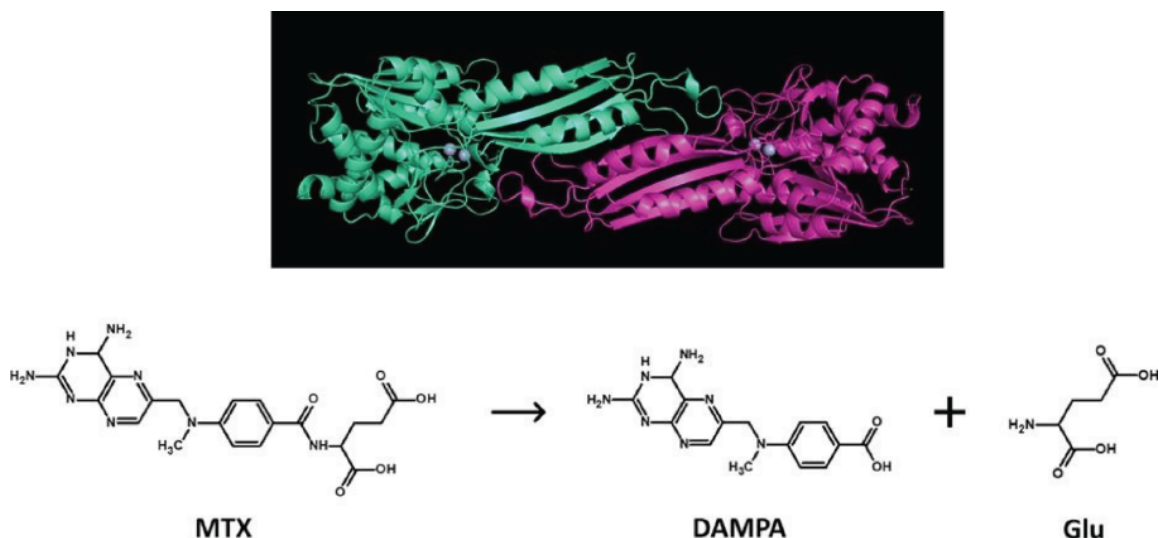


Fig. (2). Three-dimensional structure of CG2 from *Pseudomonas sp.* strain RS-16 (PDB id: 1CG2) and reaction catalyzed by the enzyme on MXT with the release of DAMPA and Glu. The two monomers of CG2 are shown in green and pink, respectively. The zinc ions within the active sites are shown as grey spheres. . (A higher resolution / colour version of this figure is available in the electronic copy of the article).

lism, peripheral arterial disease, and deep vein thrombosis are currently the main cause of death in the world [179]. These conditions mainly result from occlusion of the vascular system by blood clot formation, which prevents or slows down blood circulation [180]. Proper blood circulation depends on the equilibrium between two processes, coagulation and fibrinolysis. Blood coagulation works to reduce blood loss resulting from tissue injuries. It is activated and regulated by a cascade of reactions catalyzed largely by serine proteases, which ultimately result in the conversion of the soluble protein fibrinogen into insoluble fibrin, responsible for blood clot formation [181]. Fibrinolysis is activated simultaneously with the coagulation process to remove excess hemostatic plug when vascular repair is completed and consists of the proteolytic degradation of the fibrin network by the serine-protease plasmin (Plm) [182]. Plm is produced in the form of an inactive zymogen called plasminogen (Plg). Conversion of Plg into Plm depends on the plasminogen activator (PA) action. Plg is secreted from endothelial cells as a single polypeptide chain of 791 residues, glycosylated in four positions, and phosphorylated on Ser578. Its molecular structure consists of a Pan/apple domain, five kringle (K1-K5) domains, a flexible linker, and a serine protease-like domain. Lysine binding sites (LBS) in the K domains allow Plg to bind fibrin or other substrates with amino-terminal groups, i.e. fibrinolysis inhibitors such as ϵ -aminocaproic acid and tranexamic acids [183]. In addition, physiological PAs, namely tissue-type plasminogen activator (tPA) and urokinase-

type plasminogen activator (uPA), bind to LBS of Plg. tPA and uPA belong to the serine-protease family. They catalyze the conversion of Plg to Plm by the cleavage of the peptide bond between Arg561 and Val562. Consequently, Plm consists of two polypeptide chains, joined by two disulfide bridges, with the catalytic domain located in the C-terminal light chain [184]. In the plasma environment, in the presence of Cl^- ions, Plg assumes a closed structure. In this structure, the interaction of some positively charged amino acids located in the Pan/apple domain with the LBS of K5 domain leads to the insertion of the flexible linker between the K3 and K4 domains preventing the proteolytic attack by PAs on the activation sequence. Proteolytic removal of the Pan/apple domain (1-77 residues) allows Plg to assume the open activatable conformation, in which Arg561-Val562 is exposed to PA action [185]. The change of Plg from closed to open conformation is also caused by binding to fibrin [186]. This mechanism favors the activation of clot-localized Plg compared to free Plg. Conversion of Plg to Plm is also controlled by the action of PA inhibitors, such as PAI-1 and PAI-2. Furthermore, plasmin in the bloodstream is rapidly neutralized by circulating inhibitors such as α 2-antiplasmin and α 2-macroglobulin [187]. Studies that contributed to deepening the mechanism and regulation of Plg activation were reviewed [187, 188]. In addition to the physiological PAs, tPA and uPA, conversion of Plg to Plm can be catalyzed by several proteins synthesized by both eukaryotic and prokaryotic organisms [189, 190]. Some of these pro-

teins are used, or proposed to be used, in the clinical treatment of CDVs. Currently, the management of thrombus-occlusive diseases largely depends on fibrinolytic enzymes, which are the only agents capable of destroying pre-existing thrombi. Among them, plasminogen activators and direct-acting thrombolytic agents are reviewed in the following sections.

4.1. Plasminogen Activators

4.1.1. Plasminogen Activators Currently Used in the Clinical Treatment of CVDs

At present, fibrinolytic agents approved for therapeutic use are streptokinase (SK), uPA, and three recombinant forms of tPA: alteplase, tenecteplase, and reteplase.

SK was the first protein with fibrinolytic activity to be isolated. It is produced by several strains of *Streptococcus haemolyticus* to overcome host defenses and favor bacterial proliferation. It is a non-glycosylated, monomeric protein of 414 amino acids organized in three domains, the α -, β -, and γ -domain. Unlike physiological PAs, SK is not a serine protease and the activation of plasminogen is obtained by an indirect mechanism. The protein has two independent Plg binding sites, one constituted of residues 1-59 in the α -domain and the other of residues 143-293 in the β -domain [191]. One Plg molecule binds to the SK β -domain forming an “activator complex” that effectively catalyzes the conversion to Plm of another molecule of Plg bound in the α -domain [192]. The half-life of the circulating SK/Plg complex is about 20 minutes, as Plm once formed, exercises its proteolytic action on SK, degrading it. The binding of SK to Plg or Plm is poorly dependent on fibrin [193, 194] and, consequently, SK promotes systemic fibrinolysis rather than targeting the blood clot, with a high risk of bleeding complications [183]. Also, being produced by organisms that are evolutionarily very distant from humans, SK stimulates a strong immune response [192] that prevents the treatment from being repeated. As streptococcal infections are quite common, antibodies against streptokinase are present in most of the population [195] and contribute to decreasing the SK half-life. Various mutated or engineered SK forms have been designed to increase half-life, reduce immunogenicity, and enhance SK affinity for the fibrin-bound Plg [192]. The history of SK, from its discovery to clinical applications has been exhaustively reviewed [196]. SK efficacy in acute myocardial infarction (AMI) management was established by the GISSI study in the second half of the 1980s [197]. Currently, SK is used especially in

developing countries because it is cheaper than other thrombolytic agents are. Therapeutic indications for SK use in CVDs are reported in Table 2.

Anistreplase or anisoylated plasminogen-streptokinase activator (APSAC) is a chemically modified version of the SK/Plg complex [204], designed to circumvent the main drawbacks of SK, namely the systemic activation of Plg resulting in a high risk of bleeding and high antigenicity. It is an equimolar complex of SK and Lys77-Plg (Plg devoid of the peptide 1-77), in which Plg is reversibly inactivated by acylation in the active site [205]. The acyl group is removed from the bloodstream after intravenous administration and the complex regains its activity. The complex shows high stability in plasma with a longer half-life than other PAs, and it can be administered in a single bolus. The long half-life (90-105 min) allows the administration time to be reduced from 60 minutes for SK to 2-5 min for APSAC [205]. Moreover, APSAC maintains the immunogenic properties of SK and it must not be administered repeatedly. APSAC is approved by FDA for AMI, PE, arterial thrombosis, and deep vein thrombosis [206], but currently, it is not commonly used [190].

Fibrinolytic activity in normal urine was described for the first time in 1947 [207]. Subsequently, the agent responsible for fibrinolysis was isolated and named urokinase or urokinase-type plasminogen activator (uPA). In addition to kidney cells, uPA is produced by several types of normal tissue cells, such as endothelial and epithelial cells, fibroblasts, monocytes, and macrophages. It is primarily involved in biological processes such as cell migration and normal tissue remodeling [208]. Because of its involvement in this type of processes, uPA is actively synthesized by cancer cells and plays an active role in tumor development, thus it is also used as a tumor marker [208]. Urokinase is produced as a glycosylated single polypeptide chain (pro-uPA) consisting of 411 amino acids organized in an EGF-like domain, a kringle domain devoid of LBS (therefore with low fibrin affinity), and a serine-protease catalytic domain. The single-chain form, pro-uPA, with very low activity, activates only the Plg bound to fibrin [187]. Activation of the almost inactive pro-uPA to the fully active enzyme occurs primarily after binding of pro-uPA to the uPA-receptor (uPAR) on the cell surface [209] and depends on the cleavage of Lys158-Ile159 peptide bond by neighboring plasmin or other serine-proteases such as kallikrein. Consequently, the conversion of Plg into Plm by uPA is mainly localized on the cell surface. The uPA active form consists of two polypeptide chains, the N-terminal

Table 2. Therapeutic management of the most used thrombolytic agents.

Protein	Therapeutic Indications	Method of Administration and Posology
Streptokinase[198, 199]	Acute myocardialinfarction	Systemic intravenous (IV) infusion of 1.5 million IU over 60 min; or Local intracoronary administration of a bolus of 20000 IU followed by infusion from 2000 to 4000 IU/min over 30 to 90 min
	Acute massive pulmonaryembolism	IV infusion of 1.5 million IU over 60 to 120 min; or IV infusion of 250000 IU over 30 min followedby a maintenance infusion of 100000 IU/hour for24 h
	Occlusion of peripheral arteries (acute, sub-acute orchronic ^a)	Local catheter-based intra-arterial infusion as: <u>Gradual infusion</u> : 1000 to 2500 IU over 3 to 5min over a maximum of 250000 IU in 10 h; or <u>Prolonged continuous low-dose infusion</u> : 5000to 10000 IU/h for up to 5 days maximum
	Extensive deep veinthrombosis ^b	IV infusion of 250000 IU over 30 min, followed by maintenance infusion of 100000 IU/h for 24h
	Central retinal venous orarterial thrombosis	IV infusion of 250000 IU over 30 min followed by maintenance infusion of 100000 IU/h over 12 h
Urokinase [200]	Extensive acute proximaldeep vein thrombosis	IV infusion 4400 IU/kg over 10 min followed by4400 IU/kg/h over 12 to 24 h
	Acute massive pulmonaryembolism	IV infusion in a peripheral vein of 4400 IU/kg over 10 min followed by 4400 IU/kg/h for 12 h; or Bolus injection into the pulmonary artery of 15000 IU/kg repeated for up to 2 times at 24 h interval.
	Acute occlusive peripheral arterial disease with limb-threatening ischemia	4000 IU/min should be infused into the clot with angiographic monitoring of treatment progress. Usually, a dose of 500000 IU over 8 h should be sufficient to dissolve the clot completely.
	Thrombosed intravascularcatheters and cannulae	5000 to 25000 IU dissolved in the volumesolvent required to completely fill the lumen of catheter or cannula and locked for 20 to 60 min
Alteplase[201]	Acute myocardialinfarction	90 min (accelerated) dose regimen (treatmentwithin 6 h after symptom onset): - Patients ≥ 65 kg: IV bolus of 15 mg followedby IV infusion of 50 mg over 30 min immediately followed by IV infusion of 35 mg over 60 min; - Patients < 65 kg: IV bolus of 15 mg followedby IV infusion of 0.75 mg/kg over 30 min immediately followed by IV infusion of 0.5 mg/kg over 60 min
		3 h dose regimen (treatment between 6 to 12 hafter symptom onset): - Patients ≥ 65 kg: IV bolus of 10 mg followedby IV infusion of 50 mg over the first hour immediately followed by IV infusion of 40 mg over 120 min; - Patients < 65 kg: IV bolus of 10 mg followedby IV infusion of 1.5 mg/kg over 3h
		- Patients ≥ 65 kg: IV bolus of 10 mg followedby IV infusion of 50 mg over the first hour immediately followed by IV infusion of 40 mg over 120 min; - Patients < 65 kg: IV bolus of 10 mg followedby IV infusion of 1,5 mg/kg over 3h
	Acute massive pulmonaryembolism	- Patients ≥ 65 kg: IV bolus of 10 mg followedby IV infusion of 50 mg over the first hour immediately followed by IV infusion of 40 mg over 120 min; - Patients < 65 kg: IV bolus of 10 mg followedby IV infusion of 1,5 mg/kg over 3h
	Acute ischemic stroke ^c	The recommended total dose is 0.9 mg/kg (maximum dose 90 mg) administered as an initial IV bolus with 10% of total dose followed by IV infusion of the remainder dose over 60min
	Acute myocardialinfarction	IV single bolus injection over around 10 s. The administered dose depends on patient body weight (bw) from 30 mg for bw < 60 kg to 50mg for bw ≥ 90 kg
Tenecteplase[202]	Acute myocardialinfarction	IV single bolus injection over around 10 s. The administered dose depends on patient body weight (bw) from 30 mg for bw < 60 kg to 50mg for bw ≥ 90 kg
Retepase[203]	Acute myocardialinfarction	Two bolus injections of 17.5 mg eachadministered over 2 min. The second bolus should be administered 30 min after the first

^a not older than 6 weeks^b arterial occlusions not older than 8 hours, venous occlusions not older than 10 days^c treatment must be started as soon as possible within 4.5 h after onset of stroke symptoms

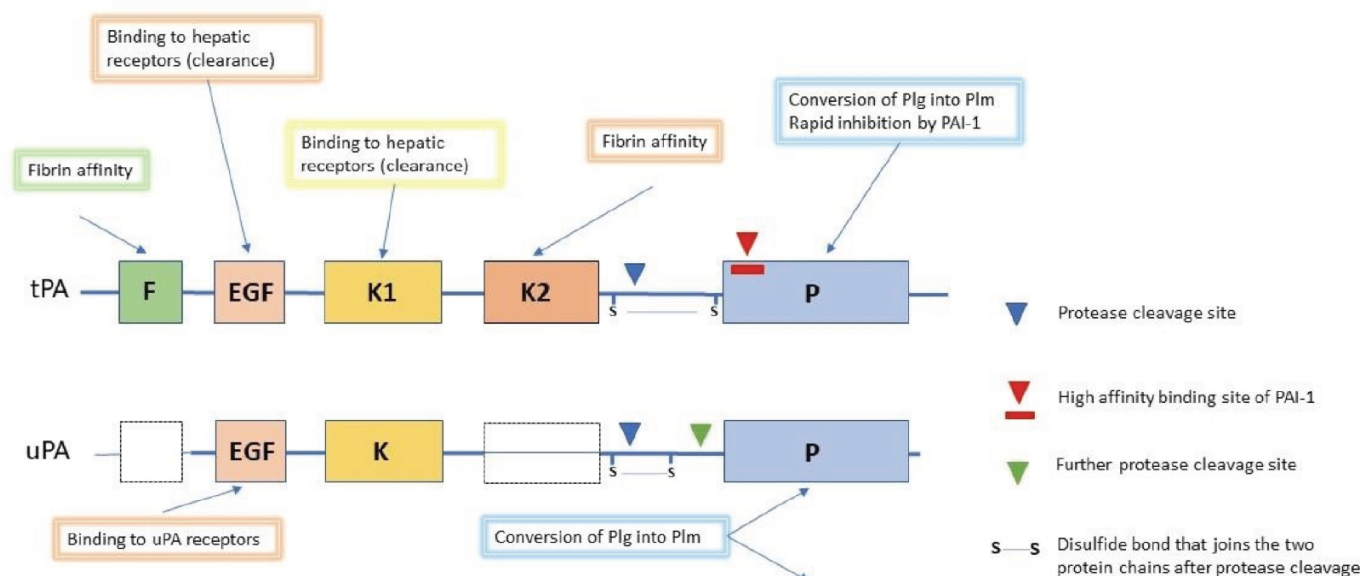


Fig. (3). Comparison of domain organization in tissue-type Plasminogen Activator (tPA), urokinase-type Plasminogen Activator (uPA), and Desmoteplase (DS). The corresponding domains are identified by the same letter (finger domain, F; epidermal growth factor domain, EGF; kringle domains, K; serine protease domain, P). Main functions of several domains are indicated. Dashed shapes indicate missing domains. . (A higher resolution / colour version of this figure is available in the electronic copy of the article).

chain with the EGF-like and kringle domains and the C-terminal chain with the serine-protease catalytic domain, joined by a disulfide bond (Fig. 3). A further proteolytic cleavage between Lys135 and Lys136 separates the two chains producing a low molecular weight (Mw ~ 33 kDa) form of uPA, mainly responsible for fibrinolytic activity in urine, and an amino-terminal fragment (ATF) which maintains the ability to bind uPAR with very high affinity [209, 210]. PAI-1 binds to uPAR/uPA complex inhibiting the proteolytic activity of uPA and favoring internalization of the complex with subsequent degradation of uPA and recycling of the receptor on the cell surface [211]. The activity of uPA is 100-fold greater than that of pro-uPA, but the lack of affinity for fibrin results in poor involvement in fibrinolysis under physiological conditions. Currently, commercially available uPA is purified from human urine or produced by recombinant technology in human neonatal kidney cells and it is predominantly made up of the low molecular weight protein. Since it is not fibrin selective, the bleeding risk related to therapeutic use is very important. uPA has been approved by the FDA for the treatment of myocardial infarction since 1978, but stopped in 1998 for deviations from Current Good Manufacturing Practices [206]. It has been reappraised for pulmonary embolism in 2002 [206]. The

therapeutic dose and administration method are reported in Table 2. Clinical use of uPA is more limited than tPA, which appears to be the major fibrinolytic agent *in vivo*. Some authors argue instead that tPA and uPA act synergistically in fibrinolysis and, from a clinical point of view, the subsequent administration of the two drugs would be advantageous as it would allow the administration of lower doses and reduce the risk of bleeding [212]. Recently, the potential of uPA as a thrombolytic agent for the treatment of stroke has been reviewed [213].

Fibrinolytic activity associated with animal tissues was first reported in 1947 [214]. Subsequently, the protein responsible for fibrinolysis was isolated from animal tissues by using strong chaotropic agents [215]. The protein was later named tissue-type plasminogen activator (tPA). It is secreted as a single glycosylated polypeptide chain of 530 residues. The molecular structure consists of a fibronectin-like finger domain, EGF-like domain, two consecutive kringle (K1 and K2) domains, and a catalytic serine-protease domain (Fig. 3). The different domains of t-PA are involved in binding to receptors and proteins, which mediate the multiple physiological roles of tPA [183]. K1 and EGF-like domains promote the binding of tPA to hepatic receptors. Moreover, the EGF-like domain interacts with the cata-

lytic domain, stabilizing it. Fibrin-specific action of tPA depends on the high affinity for fibrin of both the finger and K2 domain. Unlike uPA, tPA has enzymatic activity even in the single-chain form even if its activity increases significantly with the formation of the ternary complex fibrin/Plg/t-PA [216]. Fibrin-bound tPA converts adjacent Plg to Plm, initiating blood clot fibrinolysis. Plm and other adjacent serine proteases remove the N-terminal tripeptide of tPA and break the Arg275-Ile276 peptide bond giving rise to a two-chain protein, tc- tPA, but this does not increase the catalytic efficiency of the tPA, especially in the presence of fibrin. Free tPA in plasma has a very short half-life (3-6 minutes) [216] because of different mechanisms, which contribute to tPA inactivation: 1) glycosyl groups on the protein favor the recognition of tPA by plasma scavenger cells [217], promoting plasma clearance, 2) tPA binding to hepatic receptors induces the liver clearance [218], and 3) binding of PAI-1 to the catalytic site causing irreversible inhibition of tPA. When tPA is complexed to fibrin clot, an increased half-life is observed [219]. Human melanoma gene of tPA was expressed first in *E. coli* [220]. Subsequently, a glycosylated and proper structured recombinant tPA was produced in the mammalian cell lines. Currently, tPA recombinant form, named recombinant tPA (rtPA) or alteplase, is produced by the expression of the human gene in stabilized Chinese hamster ovary cells and used for therapeutic treatments. Alteplase shares the properties and activities of endogenous tPA, including the very short half-life and clearance primarily mediated by the liver. It was approved by FDA for treatment of acute myocardial infarction in 1987, for pulmonary embolism in 1990, and for acute ischemic stroke in 1996 [206]. It was the first recombinant form of tPA to be approved and, so far, the only fibrinolytic drug approved for the treatment of acute ischemic stroke (AIS). Therapeutic indications for the clinical management of alteplase are summarized in Table 2. The effectiveness of thrombolytic therapy is time-dependent, and early treatment increases the likelihood of a favorable outcome. Beyond the indicated therapeutic windows, the risk associated with the treatment is greater than the benefits. In particular, AIS treatment should start as soon as possible within 4.5 h after symptom onset [221, 222]. The very narrow window significantly reduces the number of patients who can receive this therapy due to delayed hospitalization. In the current global emergency caused by COVID-19, the off-label use of tPA for the treatment of seriously ill patients may reduce mortality and contribute to pandemic management [223]. In severe cases, COVID-19 causes severe coagulopathy [224, 225] with high mor-

ality rates [226]. Extensive pulmonary microthrombosis leading to acute respiratory distress syndrome (ARDS) is one of the most important reasons for mortality [227]. Patients with severe COVID-19 related ARDS have increased thrombin generation, modest or null release of tPA, increased levels of PAI-2, fibrinogen, and D-dimers [228, 229]. In this context, the use of drugs normally considered to be of high risk such as fibrinolytic agents is justified by the high probability of death of seriously ill patients [230, 231]. Urokinase and streptokinase have been previously evaluated for the treatment of ARDS [232]. In addition, the use of nebulized streptokinase appears to improve oxygenation and lung function in ARDS [233].

Administration of alteplase to COVID-19 related ARDS patients in a very critical situation with severe breathing difficulties and refractory to anticoagulant therapy has been reported [234-236]. Improvement of respiratory parameters and oxygenation related to alteplase administration was observed, although for some patients the improvement was transient [236]. In most of the cases described, the patients recovered. No patients showed bleeding complications. Similarly, no bleeding was observed after intravenous administration of alteplase for AIS in patients with COVID-19 [237]. Presumably, the high fibrinolysis inhibition associated with the viral infection significantly reduces the hemorrhagic risk.

Tenecteplase is an alteplase variant produced by site-directed mutagenesis to improve the therapeutic performance of alteplase, i.e. to increase its half-life in the bloodstream and its affinity for fibrin. As alteplase, it is produced in CHO cells. Tenecteplase has several point mutations, such as Thr103Asn, Asn117Gln, and Lys296, His297, Arg298 and Arg299, each replaced with Ala residue. The mutation at position 103 introduces a new glycosylation site, while the mutation at position 117 eliminates one of the tPA native glycosylation sites. Substitution with Ala of the residues 296-299 reduces the affinity for PAI-1 [238]. Overall, these site-specific mutations increase tenecteplase half-life (17-20 min) and fibrin affinity [239] compared to alteplase. These features allow tenecteplase to be administered at lower doses and in a shorter time than alteplase (Table 2) with reduced bleeding risk [240].

Reteplase, another variant of alteplase, is obtained by deleting the 4-175 residues of tPA. It is produced in *E. coli* as a non-glycosylated single chain of 355 amino acids organized in two domains, K2 and serine protease domains. The catalytic activity towards Plg is comparable to that of alteplase and inhibition by

PAI-I occurs at a similar degree [241]. Reteplase half-life *in vivo* (14- 18 min) is longer than that of alteplase. However, the affinity for fibrin is much lower (5 times) than that of both alteplase and tenecteplase. This favors its penetration into the clot increasing the probability of dissolving it completely [206]. On the other hand, alteplase (and tenecteplase) action is limited to the clot surface and often does not achieve complete dissolution of the thrombus, with the risk of smaller clots returning to circulation and causing further damage.

The different characteristics of alteplase, tenecteplase and reteplase result in different indications for their clinical use for both doses and the method of administration (Table 2). The only approved therapeutic indication for both reteplase and tenecteplase is the treatment of AMI. Serious harm may be suffered by the patient from the accidental use of reteplase or tenecteplase instead of alteplase in the AIS treatment [203].

4.2. Current Strategies to Improve Thrombolytic Agents

The fibrinolytic enzymes currently exploited for thrombolytic therapy have high efficiency but also serious disadvantages such as the risk of systemic bleeding with symptomatic intracranial hemorrhages and possible re-occlusion of the vessels caused by secondary clots. Studies aimed at improving therapeutic efficiency and reducing the risks associated with thrombolytic therapy follow different strategies such as: 1) improve the selectivity for fibrin and increase the catalytic efficiency of tPA by recombinant DNA techniques; 2) investigate the fibrinolytic features and validate the therapeutic use of fibrinolytic enzymes from other sources; and 3) use of thrombolytic agents that act directly on fibrin avoiding the activation step by PAs.

4.2.1. Recombinant Variants of tPA

Several variants of alteplase have been designed to improve its efficacy and safety along with a longer half-life. The structural and functional properties of these proteins, namely duteplase [242] monteplase [243], lanoteplase [217], pamiteplase [244, 245] and amideplase [246] have been recently revised [206, 247].

4.2.2. Promising Fibrinolytic Agents from Non-human Sources

Saliva of the vampire bat *Desmodus Rotundus* has high fibrinolytic activity due to a protein that acts like a

PA [248]. The protein, called desmoteplase (DS, DSPA α 1), exists as four distinct variants, DSPA α 1, α 2, β and γ , coded by four different genes. The molecular structure of each variant is common to that of human tPA with differences consisting almost exclusively in the deletion of specific domains [249, 250]. The schematic representation of the domain organization of the DSPA α 1 variant is shown in Fig. 2. Common features to all variants are: 1) lack of the LBS in the K domain, which is, therefore, more similar to K1 than K2 domain of the tPA, 2) lack of the Plm cleavage site in position 275 of the protease domain, consequently they only exist as single-chain proteins, 3) very low intrinsic activity, which increases enormously in the presence of fibrin, and 4) extraordinary selectivity towards fibrin as compared to fibrinogen [251]. As DSPA α 1 exhibits higher fibrin selectivity than the others, it was ultimately selected for clinical development [252, 253]. The selectivity towards fibrin of DSPA α 1 is also superior to that of tPA. In fact, the constant of the activation rate of Plg measured in the presence of fibrin with respect to fibrinogen is 12800 for DSPA α 1 and 72 for tPA [251]. The long half-life (about 3 h), high fibrin selectivity, lack of systemic depletion of Plg and fibrinogen, and lack of inhibition by PAI-1 make DSPA α 1 “a thrombolytic drug optimized by natural selection” [253]. As a drug, DS could give better results than tPA especially for the treatment of AIS because it has no neurotoxic effects. Unlike expectations, clinical trials investigating the safety and efficacy of DS in patients with ischemic stroke have provided no definitive evidence of its clinical benefits over tPA administration [252, 254]. More recently, outcomes of DIAS-4 trial along with a pooled analysis of the previous DIAS- 3, DIAS-4, and DIAS-J (Japan) trials have shown that the administration of DS in AIS is safe up to 9 h after the onset of symptoms; it increases arterial recanalization, but it does not significantly improve functional recovery of patients at 3 months [255].

Staphylokinase (SAK) is produced by *Staphylococcus aureus* to promote invasion of the host tissues. The protein is smaller than streptokinase, as it is made up of only 136 amino acids. The molecular structure is a homolog to the SK α -domain [256]. Like SK, SAK can bind Plg or Plm. However, while the SAK/Plg complex is inactive, SAK/Plm exhibits enhanced substrate specificity with respect to free Plm [183]. Conversion of Plg to Plm by SAK/Plm occurs primarily on the clot surface because the complex is stabilized by the fibrin binding, which also prevents α 2-antiplasmin binding. On the other hand, in the absence of fibrin, α 2-antiplasmin binds effectively to the SAK/Plm complex,

inhibits Plm and causes SAK dissociation. The interchange between the binding of fibrin and α 2-antiplasmin promotes clot fibrinolysis compared to systemic fibrinogen depletion. Consequently, there is a very low bleeding risk associated with SAK administration. Anyway, being of heterologous origin, SAK induces the formation of antibodies, which are the main cause of its neutralization in the bloodstream. In summary, SAK is a promising fibrinolytic agent with fewer side effects than other PAs [247].

4.2.3. Direct-acting Thrombolytic Agents

Direct thrombolytic agents are enzymes able to degrade fibrin directly, without the intermediate step of Plg activation. Plm and its lower molecular weight derivatives (e.g., microplasmin, miniplasmin, deltaplasmin) have the potential to improve thrombolytic therapy by reducing the bleeding risk [257]. Systemic administration of Plm is safe as it does not cause generalized fibrinolysis, given its very short half-life in the blood (0.02 s), but it is completely ineffective for thrombolytic therapy. Local catheter-based administration, on the other hand, allows Plm to be used as a thrombolytic agent acting only at the thrombus site. After thrombus degradation, the enzyme is released into the bloodstream and quickly neutralized by α 2-antiplasmin [258]. *In vivo* animal models [259] and clinical trials [260] confirmed that Plm can be used as a thrombolytic agent without any hemorrhagic consequences. To improve its clinical effectiveness, several lower molecular weight variants of Plm have been constructed by limited proteolysis or recombinant technology. The design of low molecular weight Plm is mainly aimed at the development of drugs for the treatment of deep vein thrombosis and peripheral arterial occlusion, since these diseases require long times for the administration of thrombolytic drugs (Table 2) with a high risk of bleeding. Miniplasmin (mPlm), constituted of K5, linker, and serine protease domains, has been proven to be more efficient than alteplase to restore blood circulation in a dog model of femoral artery thrombosis [261]. Thrombolytic efficacy of mPlm has been increased by site-directed mutagenesis [262]. Another Plm variant, the microplasmin (μ Plm, ocriplasmin) consists only of the linker and serine protease domains. Since it lacks all K domains, it has no affinity for fibrin and a very low affinity for α 2-antiplasmin [263]. Its main inhibitor is α 2-macroglobulin [257]. Its half-life in blood is 4 s [263]. *In vivo* animal models and clinical trials proved that μ Plm was not causing hemorrhage [264]. Site-specific PEGylation experiments to increase μ Plm stability have recently been

designed [265]. Microplasmin (under the name of ocriplasmin) has been approved by the FDA for the treatment of vitreomacular adhesion, as it causes the detachment of fibronectin and laminin [266- 268]. The use of μ Plm has also been proposed to eliminate retinal vein occlusion using a robotic micromanipulator [269, 270]. Deltaplasminogen is a deletion mutant of Plg only consisting of K1 and serine protease domains expressed in *E. coli*. It is converted to the active form, deltaplasmin, by PA action. Deltaplasmin has fibrinolytic efficacy comparable to Plm and is inhibited by both α 2-antiplasmin and α 2-macroglobulin [271].

Nattokinase (NK) is a fibrinolytic enzyme produced by *Bacillus subtilis* (*natto*), discovered in a typical Japanese cheese-like food, termed Natto, obtained by soybean fermentation [272]. NK is a 27-kDa serine-protease devoid of disulfide bonds belonging to the subtilisin family. Its fibrinolytic activity appears to be due to multiple actions [190]. In fact, NK exhibits a direct proteolytic action on fibrin, catalyzes the conversion of Plg to Plm like the PAs, increases the tPA release from human cells [273], neutralizes the PA primary inhibitor, PAI-1 [274], and prevents platelet aggregation. As compared to Plm, it exhibits an increased substrate selectivity towards fibrin with respect to fibrinogen [275]. Furthermore, NK is sufficiently stable in the gastrointestinal tract to be considered potentially useful for oral thrombolytic therapy [276, 277]. Clinical trials proved that NK administration: 1) decreases the levels of fibrinogen, factor VII, and factor VIII in plasma [278]; 2) enhances fibrinolysis [279]; and 3) reduces blood pressure and von Willebrand factor, a cardiovascular risk marker, in hypertensive patients [280]. Furthermore, the efficacy of NK as a nutraceutical adjuvant in the rehabilitation of patients with ischemic stroke has been reported [281]. The pharmacological actions of NK related to the prevention and treatment of cardiovascular diseases have been reviewed [282].

5. OTHER INDICATIONS FOR EXOGENOUS ENZYMES IN HUMAN THERAPY

5.1. Phenylketonuria

Phenylketonuria (PKU) is an autosomal recessive disease due to either a deficiency of phenylalanine hydroxylase (PAH, EC 1.14.16.1), the enzyme that converts the amino acid phenylalanine to tyrosine, or, less often, to a deficiency of its cofactor, tetrahydrobiopterin (BH4) [283, 284]. Hundreds of mutations of the PAH gene on chromosome 12 are known to cause disease. Affecting about 1 in 10000 to 15000 people in

the US, PKU is likely the most common inborn error of amino acid metabolism, and the first one for which a perinatal screening assay (the Guthrie test) has been introduced into clinical practice [285, 286].

The accumulation of dietary phenylalanine, that competes with other large neutral amino acids for transport across the blood-brain barrier (and decreased availability of tyrosine, a precursor of hormones and dopaminergic neurotransmitters) disrupts neurotransmitter balance and has toxic effects on the central nervous system, causing, when untreated, chronic neurodevelopmental and psychiatric effects. Symptoms include microcephaly, musty smell and light skin in infants, seizures, and cognitive, behavioral and motor disorders. Newborns may be normal at birth because phenylalanine is metabolized by the mother, but phenylalanine levels above the physiological range in pregnant female patients lead to adverse fetal effects [283]. If PKU is diagnosed early enough, dietary control (a diet poor in phenylalanine, supplemented with amino acid formulas) is often sufficient to prevent irreversible brain damage. However, issues related to scarce patient compliance for low diet palatability, and vitamin deficiencies, may lead to suboptimal outcomes [284]. Co-factor therapy, approved in 2007, is more effective in patients with mild forms of PKU, and less effective in the classical form of the disease [283].

While gene therapy is currently investigated for PKU treatment, enzyme substitution therapy was recently approved for adult patients with uncontrolled phenylalaninemia (greater than 600 micromol/L) on current management [287, 288]. PEGvaliase-pqpz (Palinziq®, Biomarin Pharm.) is a recombinant phenylalanine ammonia lyase (PAL, EC 4.3.1.24, formerly EC 4.3.1.5) enzyme derived from the filamentous cyanobacterium *Anabaena variabilis* that converts phenylalanine to ammonia and a harmless metabolite, trans-cinnamic acid. As for several other biopharmaceuticals [289-293], conjugation with PEG of PEGvaliase was exploited to reduce immunogenicity and improve pharmacodynamic stability. The peculiarity of this approach is that enzyme function is not replaced by the implementation of the missing enzyme (PAH), but by another functionality. In this case, the use of PAL allowed overcoming many of the shortcomings associated with the possible use of PAH itself, from its instability and difficult expression and purification, to the requirement of a cofactor, BH₄ [294]. Homotetrameric *A. variabilis* PAL has a catalytic activity comparable to that of the plant and yeast enzymes, with the advantage of being significantly smaller [295].

Both the U.S. FDA and EMA approved PEGvaliase for the treatment of adult patients with PKU in 2018. However, Palinziq, that is distributed in the form of a once-daily self-administered therapy in a single-dose prefilled syringe for subcutaneous use, is available only through a restricted program under a REMS (Risk Evaluation and Mitigation Strategy) called the Palinziq REMS, because of the risk of anaphylaxis. Other reported adverse effects include injection site reactions, arthralgia, hypersensitivity reactions, headache, generalized skin reactions, gastrointestinal symptoms, pruritus, diarrhea, nasal congestion, fatigue, dizziness, and anxiety.

Studies are ongoing to engineer PAL mutants with enhanced activity [296] or develop orally administered probiotics expressing recombinant PAL from *Photobacterium luminescens* [297]. Further insight into PKU enzyme therapy can be found in the review by Marchetti *et al.* in this CMC Special Issue.

5.2. Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disease due to mutations of both alleles of the gene coding for a chloride-conducting transmembrane channel called the cystic fibrosis transmembrane conductance regulator (CFTR) [298-301]. Despite improvements in the last decades in early diagnosis and management, with a birth prevalence of around 1:3000 in populations of northern European descent, CF is the most common lethal genetic disease in the white population [300], mostly because of lung problems. Life expectancy in developed countries is of about 40-50 years [302, 303]. More than 70000 people worldwide live with CF.

The CFTR is an ABC transporter ion channel conducting chloride ions across epithelial cell membranes, which regulates anion transport and mucociliary clearance in the airways. CFTR structure comprises five domains: two helical transmembrane domains, each connected to a nucleotide-binding domain in the cytoplasm, and a regulatory domain connecting the first nucleotide-binding domain to the second transmembrane domain [304]. ATP-driven phosphorylation of the regulatory domain opens the channel allowing anion flux, while in CF, chloride efflux out of the cell is hampered.

Although more than 1500 mutations of the CFTR gene have been identified [301, 305], at last, two-thirds of mutated alleles in northern European and North American populations are accounted for by the deletion of phenylalanine at position 508 [300]. This mutation causes incorrect folding and transport of CFTR to

the cell membrane; other mutations may cause truncated or unstable variants, or defects of ATP binding or ion transport function. Defects in the CFTR gene result in abnormal chloride conductance on the apical membrane of the epithelial cell that, in the lung, causes airway surface liquid depletion, ciliary collapse and decreased mucociliary transport. Mucus retention clogs the airways and triggers a vicious circle of chronic inflammation and infection, which may end up causing bronchiectasis and respiratory failure. Lung symptoms include breathing difficulty, cough and mucus emission, caused and/or complicated by frequent lung infections by normally commensal bacteria, and reduced exercise capacity. Clinical signs of CF regard mostly the lungs, but also other organs, like liver, kidneys, pancreas, sweat glands, vas deferens, and intestine, and may include poor growth (buildup of mucus in the pancreas prevents the release of digestive enzymes, causing malnutrition), and male infertility.

While research on a gene therapy is ongoing, the most recent therapeutic approaches for CF include CFTR modulators: potentiators that aim to improve the activity of the channel, correctors supporting proper protein traffic to the cell surface, and readthrough agents that restore full-length CFTR by suppression of premature termination codons [306-309]. The disease is also managed through antibiotic treatment of lung infections (lung transplantation in extreme cases), inhaled pancreatic saline and salbutamol, pancreatic enzyme replacement, fat-soluble vitamins supplementation, and physiotherapy [308], and through the administration of deoxyribonuclease [310-313].

Dornase alfa (Pulmozyme®, Genentech Inc.) is a highly purified inhalation solution of recombinant human deoxyribonuclease I (EC 3.1.21.1), expressed in CHO cells, that selectively cleaves extracellular DNA to 5'-phosphodinucleotide and 5'-phosphooligonucleotide end products without affecting intracellular DNA. The ca. 30kD-enzyme is folded into a four-layer α/β sandwich, an architecture typical of all members of the DNase I-like structural superfamily [314] and its optimal activity is dependent on the presence of divalent cations such as calcium and magnesium. The proposed catalytic mechanism suggests the occurrence of a magnesium-assisted pentavalent phosphate transition state, and key catalytic residues appear to be Asn7, through stabilization of the magnesium ion coordination sphere, and Asp168, which may play a key role as a general catalytic base [314].

Dornase alfa, an orphan drug [315] first marketed at the end of 1993, hydrolyzes the DNA present in the

mucus reducing its viscosity [316], and thus facilitating its clearance. Also because of limited systemic absorption upon inhalation, adverse reactions are rare and usually mild and transient. These include chest pain, fever, dyspepsia, voice alteration, pharyngitis, dyspnea, laryngitis, rhinitis, decreased lung function, rash, urticaria, and conjunctivitis (<https://go.drugbank.com/drugs/DB00003>).

Dornase alpha has also been shown to be effective in treating pathologies other than CF, like atelectasis in pediatric patients [317, 318]. Recently, it has been proposed to exploit the capability of Dornase to dissolve neutrophils extracellular traps (NETs, web-like structures of DNA and proteins expelled from the neutrophil that entrap bacteria [319, 320]) to help resolve mucus secretion in COVID-19 patients, improving ventilation and reducing the risk of secondary infections [321].

AIR DNase, alidornase alfa (PRX-110) (Protalix BioTherapeutics Inc.), a plant **cell-derived** recombinant human deoxyribonuclease I resistant to actin inhibition, is currently undergoing Phase II Clinical Trials (ClinicalTrials.gov identifier: NCT02722122).

5.3. Lactose Intolerance

Lactose intolerance (LI) is a common condition caused by lactase deficiency. The most common form of LI is the genetically determined adult-onset lactose maldigestion due to loss of intestinal lactase (EC 3.2.1.108, AKA lactase/phlorizin hydrolase for its activity on 3-glycosylceramides), an integral membrane hydrolase produced by the enterocytes lining the walls of the small intestine that form the brush border [322]. The ability to retain lactase into adulthood is a dominant trait [323] and divides the world human population into two phenotypes, lactase persistent and non-persistent. Lactose malabsorption and colonic fermentation cause primarily gastrointestinal symptoms like bloating, flatulence, abdominal discomfort, and diarrhea. Apart from dietary interventions, LI can be treated with oral administration of prebiotics, probiotics, or exogenous lactase, produced from fungi or yeasts. Indeed, the term lactase includes several enzymes capable of **catalyzing** the hydrolysis of the lactose disaccharide into the constituent monosaccharides D-galactose and D-glucose. Among these, the human β -galactosidase has a tetrameric quaternary structure; the mature monomer is characterized by **an** MW of 160 kDa [324] and contains two catalytic glutamate residues.

Several studies reviewed [325-327], report on the efficacy of oral administration of probiotics with β -galactosidase activity on gastrointestinal symptoms and hydrogen breath excretion. As for the administration of exogenous lactase (in form of gels, liquids, tablets, or capsules), alone or in association with probiotics, randomized clinical trials reported by Portincasa *et al.* [328], [329] and De Vrese *et al.* [330] indicated that tilactase, a β -D-galactosidase from *Aspergillus oryzae*, strongly improves both hydrogen breath excretion and gastrointestinal symptoms after lactose ingestion by lactose intolerants. However, more recent studies yielded less conclusive results [331], and additional investigations were recommended to better define the efficacy of exogenous enzymes on LI [327]. In 2009, the European Food Safety Authority (EFSA) released a Scientific Opinion according to which “a cause and effect relationship has been established between the consumption of lactase enzyme and breaking down lactose in individuals with symptomatic lactose maldigestion” [332], and several formulations of lactase are commercially available as dietary supplements.

5.4. Chemonucleolysis

Disc herniation, consisting of protrusion of the inner part of a spinal disc (nucleus pulposus) out of a damaged outer part (annulus fibrosus), resulting in nerve root compression, is most often treated with conservative therapy. Starting from the 1980s, chemonucleolysis with the chymopapain protease was often exploited as an alternative or intermediate treatment before surgical therapy [333, 334]. Chymopapain (EC 3.4.22.6) is an extracellular plant cysteine proteinase similar to papain in specificity [335], with a homodimeric quaternary structure. The active site, containing the Cys25, His159, and Asn179 catalytic triad, is located at the interface between the two domains of each protomer, that are similar in size but endowed with different structure: an L-domain, mainly α -helical, and an R-domain essentially consisting of extensive antiparallel β -sheet interactions [336].

Chymopapain intradiscal injection has the purpose to degrade proteoglycans of the nucleus pulposus without damaging the annulus, thus causing disc shrinkage and reducing compression on adjacent nerves [333, 337, 338]. Chymopapain was FDA-approved in 1982 and later withdrawn from sale (in 2003), although apparently for commercial, and not for safety or effectiveness reasons. However, it has to be noted that adverse effects like anaphylaxis, bleeding and neurological complications were associated with the use of chymopapain for chemonucleolysis [339-341].

As an alternative to chymopapain, several enzymes were considered for chemonucleolysis [342], but only a few were used in clinical settings: collagenase (see section 5.5, in this review) and condoliase.

Collagenase was reported to cause fewer adverse effects, and also to be clinically less effective with respect to chymopapain [343-345].

Condoliase (chondroitin sulfate ABC endolyase from *Proteus vulgaris*, EC 4.2.2.20) is a glycosaminoglycan-degrading enzyme that cleaves the β -1,4-galactosaminic bonds between N-acetylgalactosamine and either D-glucuronic acid or L-iduronic acid. This endolyase has an elongated three-domain structure [346], comprising an N-terminal domain with a fold similar to that of carbohydrate-binding domains of xylanases and some lectins, a substrate-binding middle domain with an $(\alpha/\alpha)_5$ fold and a C-terminal domain with β -sheet folding. *P. vulgaris* chondroitin sulfate ABC endolyase is endowed with remarkably broad specificity, degrading chondroitin, chondroitin-4-sulfate, dermatan sulfate, chondroitin-6-sulfate, and hyaluronan. Its proposed use in the treatment of central nervous system degeneration further raised interest in the enzyme [347], and led to recent efforts to increase its stability and efficacy [348].

Chondroitin sulfate is present in several tissues as skin, bones, cartilage and the nucleus pulposus. However, with condoliase being more specific than chymopapain or collagenase, it is likely to be less harmful to the nervous tissue and other structures, except cartilage and the nucleus pulposus itself [342]. Clinical trials on the treatment of lumbar disc disease, lumbar vertebral hernia, and intervertebral disc disease [334, 349, 350] showed a significant improvement in the primary endpoint (change in the worst leg pain at 13 weeks after a single administration in the Chiba *et al.* study [349]). Following these Phase III trials, condoliase (Herni-core®, Seikagaku Co., Tokio, Japan) was approved in 2018 by the drug regulatory authority in Japan for intradiscal therapy of lumbar disc herniation, and recently proposed also for the treatment of cervical disc herniation [342].

5.5. Collagenolysis

Collagenases are multidomain enzymes that catalyze the hydrolysis of collagen thus leading to the loosening of the connective tissue structure. In mammals, the activity of collagenase is linked to tissue remodeling, embryo development and wound healing [351-353]. In bacteria, collagenases are involved in both tissue penetration and invasion and nutrient release.

Collagenases identification, characterization and classification are still largely in their infancy and much has still to be done to improve our ability to fully exploit the potential of these enzymes. Indeed, collagenases are especially susceptible to incorrect classification. Often, proteolytic enzymes classified as collagenases are indeed general peptidases or gelatinases [354]. True collagenases are proteases that cleave fully folded collagen molecules or collagen fibrils, whereas gelatinases or non-specific peptidases are proteolytic enzymes that cleave (partially) denatured collagen. Human collagenases are better characterized than their bacterial counterparts ~~are~~ and show a very strict substrate specificity. Human collagenases cleave tropocollagen at position 775/776 and produce two fragments, a longer one, named TCA and a shorter one, named TCB [353, 355, 356]. They ~~and~~ show different specificities for different collagen types. Microbial collagenases (EC 3.4.24.3) are less specific than animal collagenases, both for the type of collagen and for the site of cleavage. Bacterial collagenases are usually more active than **their** mammalian counterpart, making these enzymes more suitable for biotechnological applications. Among bacterial collagenases, those expressed by clostridia are the most efficient. Collagenases from *Clostridium histolyticum* were isolated, characterized and classified in the 1980s by Bond and Van Wart [357-359]. *C. histolyticum* expresses seven different collagenases that have different functional, biochemical, and biophysical properties, the most relevant of which is their substrate preference. Bacterial collagenases are zinc-dependent metalloproteases that also require calcium ions for proper function. Class I collagenases, which include α , β , γ and η isoforms, have high collagenolytic activity and moderate peptidolytic activity, as determined using the synthetic peptide 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine. Class II collagenases, which include δ , ϵ , and ζ isoforms, have moderate collagenolytic activity and high peptidolytic activity [357]. Both type I and type II collagenases cleave at the Xaa↓Gly-Pro-HyP site on the substrate [360], but type I collagenases attack close to the N- and C-termini of type I human collagen giving one larger fragment, whereas type II collagenases attack a single site inside the molecule giving two smaller fragments [361]. The initial cleavage is followed by subsequent lytic events that cleave the substrate in smaller peptides. Two genes that possibly evolved from a gene duplication event followed by divergent evolution [359] code for class I and class II collagenases. In *C. histolyticum*, *colG* codes for the 126-kDa class I collagenase and *colH* for the 116-kDa class II collagenase.

Smaller forms are then produced by the C-terminal proteolytic cleavage of the full-length precursors. Indeed, all the seven isoforms share the same N-terminal sequence [362]. ColG and ColH from *C. histolyticum* share a common multi-domain modular structure (Fig. 4) with an N-terminal activator domain, a central peptidase domain and C-terminal polycystic kidney disease-like (PKD) and collagen binding (CBD) domains. ColG has one PDK-like domain and two CBDs, whereas ColH has two PDK-like domains and one CBD [356]. Crystal structures of isolated domains or larger fragments have been solved and allowed to propose a “chew-and-digest” mechanism for collagen recognition and trimming [363]. The activator domain is essential to direct specifically the protease activity towards collagen; the isolated peptidase domain is active on synthetic, short substrates but is inactive on full-length collagen. The activator and catalytic domains form a saddle-shaped structure that, upon substrate binding, shifts from an open conformation to a closed one (Fig. 4). **In closed conformation** both the activator and the catalytic domains contact the substrate, that can be either a collagen triple helix or a microfibril [363]. In the closed state, the activator domain is responsible for helix unwinding that allows the proteolytic cleavage of one helix at a time by the catalytic domain. Triple helix unwinding is energetically assisted by substrate desolvation [363] and by a calcium-triggered switch in the CBD [364].

Bacterial collagenases, due to their processivity and moderate substrate specificity, find many biotechnological applications, including therapeutic use in the breakdown of collagen plaques, debridement of necrotic tissue due to burns or chronic ulcers [366-368]. Recently, FDA approved a mixture of bacterial collagenases, marketed as QWO™, for the treatment of moderate to severe cellulite in the buttocks of adult women [369]. Collagenase *C. histolyticum* was approved in February 2010 by FDA for the treatment of Dupuytren's contracture [354] and in 2013 by FDA and in 2014 by EMA for the treatment of Peyronie's disease [370]. In Europe, the brand name for Collagenase *C. histolyticum* was Xiapex® but the product was withdrawn by EMA at the request of the marketing authorization holder, Swedish Orphan Biovitrum AB, which notified the European Commission of its decision to permanently discontinue the marketing of the product for commercial reasons. The withdrawal is effective as of 1 March 2020. In the U.S. and Canada, Collagenase *C. histolyticum* is marketed under the name Xiaflex® (Endo Ventures Ltd; Endo Pharmaceuticals Inc.). Both products contain a mixture of ColH

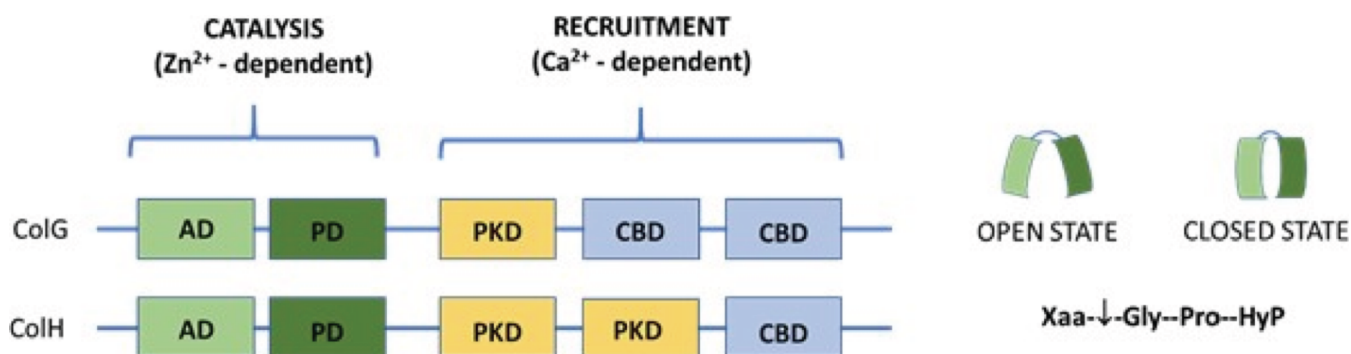


Fig. (4). ColG and ColH domain organization, structurally-identified conformational states and substrate cleavage site. Modified from [361, 365]. . (A higher resolution / colour version of this figure is available in the electronic copy of the article).

and ColG collagenases. Preparations contain 0.9 mg of a lyophilized formulation of the two collagenases co-expressed and harvested from anaerobic fermentation of a phenotypically selected strain of *C. histolyticum* [371, 372].

Breakdown of collagen plaques for the treatment of Dupuytren's contracture. Dupuytren's disease is characterized by the deposition of type I and III collagen in the hand and can show a spectrum of symptoms, from almost asymptomatic to severe hand contractures (Dupuytren's contracture)[373]. The disease is more common in Northern Europe and in men [373] and can be surgically treated. Treatment with collagenase intralesional injections represents a less invasive, well-tolerated therapeutic approach [374, 375]. In a prospective, randomized, double-blind, placebo-controlled, multicenter trial that enrolled 308 patients, up to three injections of Collagenase *C. histolyticum* were administered to patients at a dose of 0.58 mg of lyophilized enzyme per injection. 64% of patients, versus 6.8% of controls, reported a significant improvement of the full extension within 30 days from treatment and the results were stable at 90 days [375]. More than 96% of patients reported adverse effects, most of which were mild to moderate and resolved within a median of ten days. The most common mild adverse events were peripheral oedema and contusion. A small number of patients reported severe adverse events, like tendon rupture or severe pain at the site of injection.

Breakdown of collagen plaques for the treatment of Peyronie's disease. Peyronie's disease is a condition affecting 3-9% of adult males worldwide and is caused by the formation of plaques in the tunica albuginea that causes moderate to severe penile deformities. Early-stage disease is preferentially treated by the intralesional administration of *C. histolyticum* collagenase followed by penile plaque remodeling [366], whereas chronic disease usually undergoes surgical treatment.

The specificity of action of collagenases on type I and type II collagen allows specific plaque removal without affecting the surrounding tissues and vessels that are enriched with type IV collagen [366]. The clinical efficacy, safety and tolerability of collagenase for the treatment of Peyronie's disease were assessed in two large, double-blind, randomized, placebo-controlled phase-III clinical studies, Impress I (enrolling 417 subjects) and Impress II (enrolling 415 subjects) [376]. Most adverse events were mild to moderate, like penile ecchymosis and swelling, although serious events, like corporal rupture has been reported in few cases. Meaningful improvement of penile curvature and of symptom, support the efficacy of treatment of Peyronie's disease with collagenase.

Bacterial collagenase is also used in ointments for the removal of damaged tissue from wounds or burns (debridement) [368, 377]. Santyl Ointment (Smith and Nephew, Inc.) was approved by FDA as a topical formulation to be used for the debridement of necrotic tissue due to burns or chronic ulcers. A similar indication is applied to bromelain, approved by EMA in 2012 and marketed under the name Nexobrid® [378] for the removal of eschar in adults with deep partial- and full-thickness thermal burns. The preparation contains a concentrate of proteolytic enzymes from the pineapple stem enriched in bromelain (EC 3.4.22.32).

CONCLUSIONS

Therapeutic enzymes have become major players in the pharmaceutical industry by virtue of their specificity, high activity, and selectivity. Their development and production have evolved over the past decades, thanks to significant innovations in biotechnology, protein chemistry, microbiology, and material science that have offered new opportunities for tailoring their biochemical and biophysical properties and to conjugate these enzymes with a broad range of polymers and

nanocarriers for targeted delivery. In addition, the evolution of novel cloning strategies and heterologous expression platforms facilitated the unprecedented increase in their production. All these advancements helped in circumventing complications, such as the high purity required, immunogenicity and short half-life, which have often limited their adoption in the clinics.

New targets for enzyme-based treatments are constantly emerging from the ever-expanding knowledge of molecular processes involved in disease origin and evolution, and will benefit from the expansion in the diversity of catalytic activities applicable to therapy triggered by “omics” technologies.

LIST OF ABBREVIATIONS

ADC	=	L-Arginine decarboxylase	GSH	=	Glutathione
ADEPT	=	Antibody-directed enzyme prodrug therapy	HA	=	Hyaluronan
ADI	=	Arginine deiminase	IS	=	Ischemic stroke
ALL	=	Acute Lymphoblastic Leukemia	ITs	=	Immunotoxins
AMI	=	Acute myocardial infarction	LBS	=	Lysine binding sites
APSAC	=	Anisoylated plasminogen-streptokinase activator	LI	=	Lactose intolerance
ARDS	=	Acute respiratory distress syndrome	MGL	=	L- methionine γ -lyase
ARGase	=	L-Arginase	MI	=	Myocardial infarction
ASL	=	Argininosuccinate lyase	mPlm	=	Miniplasmin
ASNase	=	L-asparaginase	μ Plm	=	Microplasmin
ASS	=	Argininosuccinate synthase	MTX	=	Methotrexate
BH4	=	Tetrahydrobiopterin	NETs	=	Neutrophils extracellular traps
CBD	=	Collagen binding domain	NK	=	Nattokinase
CF	=	Cystic fibrosis	OTC	=	Ornithine transcarbamylase
CFCR	=	Cystic fibrosis transmembrane conductance regulator	PA	=	Plasminogen activator
CG2	=	Carboxypeptidase G2	PAH	=	Phenylalanine hydroxylase
CVDs	=	Cardiovascular disorders	PAL	=	Phenylalanine ammonia lyase
DAMPA	=	2,4-diamino-N10-methylpteroic acid	PE	=	Pulmonary embolism
DS	=	Desmoteplase	PEA	=	<i>Pseudomonas aeruginosa</i> exotoxin A
DT	=	Diphtheria toxin	PEG	=	Polyethylene glycol
EMA	=	European Medicines Agency	PKD	=	Polycystic kidney disease
FDA	=	Food and Drug Administration	PKU	=	Phenylketonuria
GNPs	=	Gold nanoparticles	Plg	=	Plasminogen
GOx	=	Glucose oxidase	Plm	=	Plasmin
			PLP	=	Pyridoxal 5'-phosphate
			REMS	=	Risk Evaluation and Mitigation Strategy
			RIPs	=	Ribosome inactivating proteins
			RNase	=	Ribonuclease
			ROS	=	Reactive oxygen species
			rtPA	=	Recombinant-tPA
			SAK	=	Staphylokinase
			SC	=	Succinimidyl carbamate
			tPA	=	ST-elevation myocardial infarction
			uPA	=	Urokinase-type plasminogen activator
			uPAR	=	uPA-receptor

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

- [1] Selwan, E.M.; Finicle, B.T.; Kim, S.M.; Edinger, A.L. Attacking the supply wagons to starve cancer cells to death. *FEBS Lett.*, **2016**, *590*(7), 885-907. <http://dx.doi.org/10.1002/1873-3468.12121> PMID: 26938658
- [2] Pavlova, N.N.; Thompson, C.B. The emerging hallmarks of cancer metabolism. *Cell Metab.*, **2016**, *23*(1), 27-47. <http://dx.doi.org/10.1016/j.cmet.2015.12.006> PMID: 26771115
- [3] Cantor, J.R.; Panayiotou, V.; Agnello, G.; Georgiou, G.; Stone, E.M. Engineering reduced-immunogenicity enzymes for amino acid depletion therapy in cancer. *Methods Enzymol.*, **2012**, *502*, 291-319. <http://dx.doi.org/10.1016/B978-0-12-416039-2.00015-X> PMID: 22208990
- [4] Pokrovsky, V.S.; Chepikova, O.E.; Davydov, D.Z.; Zamyatin, A.A., Jr; Lukashev, A.N.; Lukasheva, E.V. Amino Acid Degrading Enzymes and their Application in Cancer Therapy. *Curr. Med. Chem.*, **2019**, *26*(3), 446-464. <http://dx.doi.org/10.2174/0929867324666171006132729> PMID: 28990519
- [5] Lubkowski, J.; Vanegas, J.; Chan, W.K.; Lorenzi, P.L.; Weinstein, J.N.; Sukharev, S.; Fushman, D.; Rempe, S.; Anishkin, A.; Wlodawer, A. Mechanism of Catalysis by L-Asparaginase. *Biochemistry*, **2020**, *59*(20), 1927-1945. <http://dx.doi.org/10.1021/acs.biochem.0c00116> PMID: 32364696
- [6] Michalska, K.; Jaskolski, M. Structural aspects of L-asparaginases, their friends and relations. *Acta Biochim. Pol.*, **2006**, *53*(4), 627-640. http://dx.doi.org/10.18388/abp.2006_3291 PMID: 17143335
- [7] Krishnapura, P.R.; Belur, P.D.; Subramanya, S. A critical review on properties and applications of microbial L-asparaginases. *Crit. Rev. Microbiol.*, **2016**, *42*(5), 720-737. PMID: 25865363
- [8] Kidd, J.G. Regression of transplanted lymphomas induced *in vivo* by means of normal guinea pig serum. I. Course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse serum, or rabbit serum. *J. Exp. Med.*, **1953**, *98*(6), 565-582. <http://dx.doi.org/10.1084/jem.98.6.565> PMID: 13109110
- [9] Broome, J.D. Studies on the mechanism of tumor inhibition by L-asparaginase. Effects of the enzyme on asparagine levels in the blood, normal tissues, and 6C3HED lymphomas of mice: Differences in asparagine formation and utilization in asparaginase-sensitive and -resistant lymphoma cells. *J. Exp. Med.*, **1968**, *127*(6), 1055-1072. <http://dx.doi.org/10.1084/jem.127.6.1055> PMID: 4871211
- [10] Loeb, E.; Hill, J.M.; Hill, N.O.; MacLellan, A.; Khan, A.; Alexander, T.R.; Adachi, A. Treatment of acute leukemia with L-asparaginase. *Recent Results Cancer Res.*, **1970**, *33*, 204-218. http://dx.doi.org/10.1007/978-3-642-99984-0_26 PMID: 5292717
- [11] Aguayo, A.; Cortes, J.; Thomas, D.; Pierce, S.; Keating, M.; Kantarjian, H. Combination therapy with methotrexate, vincristine, polyethylene-glycol conjugated-asparaginase, and prednisone in the treatment of patients with refractory or recurrent acute lymphoblastic leukemia. *Cancer*, **1999**, *86*(7), 1203-1209. [http://dx.doi.org/10.1002/\(SICI\)1097-0142\(19991001\)86:7<1203::AID-CNCR15>3.0.CO;2-3](http://dx.doi.org/10.1002/(SICI)1097-0142(19991001)86:7<1203::AID-CNCR15>3.0.CO;2-3) PMID: 10506705
- [12] Aljewari, H.; Nader, M.; Al-Faisal, A.; Weerapreeyakul, N.; Sahapat, S. High efficiency, selectivity against cancer cell line of purified L-Asparaginase from pathogenic *Escherichia coli*. *World Acad. Sci. Eng. Technol.*, **2010**, *65*, 416-421.
- [13] Ando, M.; Sugimoto, K.; Kitoh, T.; Sasaki, M.; Mukai, K.; Ando, J.; Egashira, M.; Schuster, S.M.; Oshimi, K. Selective apoptosis of natural killer-cell tumours by L-asparaginase. *Br. J. Haematol.*, **2005**, *130*(6), 860-868. <http://dx.doi.org/10.1111/j.1365-2141.2005.05694.x> PMID: 16156856
- [14] Pieters, R.; Carroll, W.L. Biology and treatment of acute lymphoblastic leukemia. *Hematol. Oncol. Clin. North Am.*, **2010**, *24*(1), 1-18. <http://dx.doi.org/10.1016/j.hoc.2009.11.014> PMID: 20113893
- [15] Yunis, A.A.; Arimura, G.K.; Russin, D.J. Human pancreatic carcinoma (MIA PaCa-2) in continuous culture: Sensitivity to asparaginase. *Int. J. Cancer*, **1977**, *19*(1), 128-135. <http://dx.doi.org/10.1002/ijc.2910190118> PMID: 832918
- [16] Avramis, V.I.; Panosyan, E.H. Pharmacokinetic/pharmacodynamic relationships of asparaginase formulations: The past, the present and recommendations for the future. *Clin. Pharmacokinet.*, **2005**, *44*(4), 367-393. <http://dx.doi.org/10.2165/00003088-200544040-00003> PMID: 15828851
- [17] Kato, M.; Manabe, A. Treatment and biology of pediatric acute lymphoblastic leukemia. *Pediatr. Int. (Roma)*, **2018**, *60*(1), 4-12. <http://dx.doi.org/10.1111/ped.13457> PMID: 29143423
- [18] Bhojwani, D.; Yang, J.J.; Pui, C.H. Biology of childhood acute lymphoblastic leukemia. *Pediatr. Clin. North Am.*, **2015**, *62*(1), 47-60. <http://dx.doi.org/10.1016/j.pcl.2014.09.004> PMID: 25435111
- [19] Müller, H.J.; Boos, J. Use of L-asparaginase in childhood ALL. *Crit. Rev. Oncol. Hematol.*, **1998**, *28*(2), 97-113. [http://dx.doi.org/10.1016/S1040-8428\(98\)00015-8](http://dx.doi.org/10.1016/S1040-8428(98)00015-8) PMID: 9768345
- [20] Perel, Y.; Auvrignon, A.; Leblanc, T.; Vannier, J.P.; Michel, G.; Nelken, B.; Gandemer, V.; Schmitt, C.; Lamagnere, J.P.; De Lumley, L.; Bader-Meunier, B.; Couillaud, G.; Schaison, G.; Landman-Parker, J.; Thuret, I.; Dalle, J.H.; Baruchel, A.; Leverger, G.; Immunology, G.L.F.S.P.H. Impact of addition of maintenance therapy to intensive induction and consolidation chemotherapy for childhood acute myeloblastic leukemia: Results of a prospective randomized trial, LAME

- 89/91. Leucémie Aiguë Myéloïde Enfant. *J. Clin. Oncol.*, **2002**, 20(12), 2774-2782.
<http://dx.doi.org/10.1200/JCO.2002.07.300> PMID: 12065553
- [21] Willems, L.; Jacque, N.; Jacquiel, A.; Neveux, N.; Maciel, T.T.; Lambert, M.; Schmitt, A.; Poulain, L.; Green, A.S.; Uzunov, M.; Kosmider, O.; Radford-Weiss, I.; Moura, I.C.; Auberger, P.; Ifrah, N.; Bardet, V.; Chapuis, N.; Lacombe, C.; Mayeux, P.; Tamburini, J.; Bouscary, D. Inhibiting glutamine uptake represents an attractive new strategy for treating acute myeloid leukemia. *Blood*, **2013**, 122(20), 3521-3532.
<http://dx.doi.org/10.1182/blood-2013-03-493163> PMID: 24014241
- [22] Yamaguchi, M.; Kwong, Y.L.; Kim, W.S.; Maeda, Y.; Hashimoto, C.; Suh, C.; Izutsu, K.; Ishida, F.; Isobe, Y.; Sueoka, E.; Suzumiya, J.; Kodama, T.; Kimura, H.; Hyo, R.; Nakamura, S.; Oshimi, K.; Suzuki, R.; Phase, I.I. Phase II study of SMILE chemotherapy for newly diagnosed stage IV, relapsed, or refractory extranodal natural killer (NK)/T-cell lymphoma, nasal type: The NK-Cell Tumor Study Group study. *J. Clin. Oncol.*, **2011**, 29(33), 4410-4416.
<http://dx.doi.org/10.1200/JCO.2011.35.6287> PMID: 21990393
- [23] Dufour, E.; Gay, F.; Agüera, K.; Scoazec, J.Y.; Horand, F.; Lorenzi, P.L.; Godfrin, Y. Pancreatic tumor sensitivity to plasma L-asparagine starvation. *Pancreas*, **2012**, 41(6), 940-948.
<http://dx.doi.org/10.1097/MPA.0b013e318247d903> PMID: 22513289
- [24] Lorenzi, P.L.; Llamas, J.; Gunsior, M.; Ozbun, L.; Reinhold, W.C.; Varma, S.; Ji, H.; Kim, H.; Hutchinson, A.A.; Kohn, E.C.; Goldsmith, P.K.; Birrer, M.J.; Weinstein, J.N. Asparagine synthetase is a predictive biomarker of L-asparaginase activity in ovarian cancer cell lines. *Mol. Cancer Ther.*, **2008**, 7(10), 3123-3128.
<http://dx.doi.org/10.1158/1535-7163.MCT-08-0589> PMID: 18852115
- [25] Lorenzi, P.L.; Reinhold, W.C.; Rudelius, M.; Gunsior, M.; Shankavaram, U.; Bussey, K.J.; Scherf, U.; Eichler, G.S.; Martin, S.E.; Chin, K.; Gray, J.W.; Kohn, E.C.; Horak, I.D.; Von Hoff, D.D.; Raffeld, M.; Goldsmith, P.K.; Caplen, N.J.; Weinstein, J.N. Asparagine synthetase as a causal, predictive biomarker for L-asparaginase activity in ovarian cancer cells. *Mol. Cancer Ther.*, **2006**, 5(11), 2613-2623.
<http://dx.doi.org/10.1158/1535-7163.MCT-06-0447> PMID: 17088436
- [26] Pasut, G.; Sergi, M.; Veronese, F.M. Anti-cancer PEG-enzymes: 30 years old, but still a current approach. *Adv. Drug Deliv. Rev.*, **2008**, 60(1), 69-78.
<http://dx.doi.org/10.1016/j.addr.2007.04.018> PMID: 17869378
- [27] Soares, A.L.; Guimarães, G.M.; Polakiewicz, B.; de Moraes Pitombo, R.N.; Abrahão-Neto, J. Effects of polyethylene glycol attachment on physicochemical and biological stability of E. coli L-asparaginase. *Int. J. Pharm.*, **2002**, 237(1-2), 163-170.
[http://dx.doi.org/10.1016/S0378-5173\(02\)00046-7](http://dx.doi.org/10.1016/S0378-5173(02)00046-7) PMID: 11955814
- [28] Melik-Nubarov, N.S.; Grozdova, I.D.; Lomakina, G.Y.; Pokrovskaya, M.V.; Pokrovski, V.S.; Aleksandrova, S.S.; Abakumova, O.Y.; Podobed, O.V.; Grishin, D.V.; Sokolov, N.N. PEGylated recombinant L-asparaginase from *Erwinia carotovora*: Production, properties, and potential applications. *Prikl. Biokhim. Mikrobiol.*, **2017**, 53(2), 164-172.
 PMID: 29508977
- [29] Thomas, X.; Le Jeune, C. Erythrocyte encapsulated L-asparaginase (GRASPA) in acute leukemia. *Int. J. Hematol. Oncol.*, **2016**, 5(1), 11-25.
<http://dx.doi.org/10.2217/ijh-2016-0002> PMID: 30302200
- [30] Brito, A.E.M.; Pessoa, A., Jr; Converti, A.; Rangel-Yagui, C.O.; Silva, J.A.D.; Apolinário, A.C. Poly (lactic-co-glycolic acid) nanospheres allow for high L-asparaginase encapsulation yield and activity. *Mater. Sci. Eng. C*, **2019**, 98, 524-534.
<http://dx.doi.org/10.1016/j.msec.2019.01.003> PMID: 30813054
- [31] Wan, S.; He, D.; Yuan, Y.; Yan, Z.; Zhang, X.; Zhang, J. Chitosan-modified lipid nanovesicles for efficient systemic delivery of L-asparaginase. *Colloids Surf. B Biointerfaces*, **2016**, 143, 278-284.
<http://dx.doi.org/10.1016/j.colsurfb.2016.03.046> PMID: 27022867
- [32] Cantor, J.R.; Stone, E.M.; Chantranupong, L.; Georgiou, G. The human asparaginase-like protein 1 hASRGL1 is an Ntn hydrolase with beta-aspartyl peptidase activity. *Biochemistry*, **2009**, 48(46), 11026-11031.
<http://dx.doi.org/10.1021/bi901397h> PMID: 19839645
- [33] Saarela, J.; Oinonen, C.; Jalanko, A.; Rouvinen, J.; Peltonen, L. Autoproteolytic activation of human aspartylglucosaminidase. *Biochem. J.*, **2004**, 378(Pt 2), 363-371.
<http://dx.doi.org/10.1042/bj20031496> PMID: 14616088
- [34] Belviso, S.; Iuliano, R.; Amato, R.; Perrotti, N.; Menniti, M. The human asparaginase enzyme (ASPG) inhibits growth in leukemic cells. *PLoS One*, **2017**, 12(5), e0178174.
<http://dx.doi.org/10.1371/journal.pone.0178174> PMID: 28542249
- [35] Beckett, A.; Gervais, D. What makes a good new therapeutic L-asparaginase? *World J. Microbiol. Biotechnol.*, **2019**, 35(10), 152.
<http://dx.doi.org/10.1007/s11274-019-2731-9> PMID: 31552479
- [36] Al-Dulimi, A.G.; Al-Saffar, A.Z.; Sulaiman, G.M.; Khalil, K.A.A.; Khashan, K.S.; Al-Shmgani, H.S.A.; Ahmed, E.M. Immobilization of L-asparaginase on gold nanoparticles for novel drug delivery approach as anti-cancer agent against human breast carcinoma cells. *J. Mater. Res.*, **2020**, 9(6), 15394-15411.
- [37] Xiong, L.; Teng, J.L.L.; Botelho, M.G.; Lo, R.C.; Lau, S.K.P.; Woo, P.C.Y. Arginine Metabolism in Bacterial Pathogenesis and Cancer Therapy. *Int. J. Mol. Sci.*, **2016**, 17(3), 363.
<http://dx.doi.org/10.3390/ijms17030363> PMID: 26978353
- [38] Dillon, B.J.; Prieto, V.G.; Curley, S.A.; Ensor, C.M.; Holtsberg, F.W.; Bomalaski, J.S.; Clark, M.A. Incidence and distribution of argininosuccinate synthetase deficiency in human cancers: A method for identifying cancers sensitive to arginine deprivation. *Cancer*, **2004**, 100(4), 826-833.
<http://dx.doi.org/10.1002/cncr.20057> PMID: 14770441
- [39] Qiu, F.; Huang, J.; Sui, M. Targeting arginine metabolism pathway to treat arginine-dependent cancers. *Cancer Lett.*, **2015**, 364(1), 1-7.
<http://dx.doi.org/10.1016/j.canlet.2015.04.020> PMID: 25917076
- [40] Fernandes, H.S.; Silva Teixeira, C.S.; Fernandes, P.A.; Ramos, M.J.; Cerqueira, N.M. Amino acid deprivation using enzymes as a targeted therapy for cancer and viral infections. *Expert Opin. Ther. Pat.*, **2017**, 27(3), 283-297.
<http://dx.doi.org/10.1080/13543776.2017.1254194> PMID: 27813440
- [41] Bobak, Y.P.; Vynnytska, B.O.; Kurlishchuk, Y.V.; Sibirny, A.A.; Stasyk, O.V. Cancer cell sensitivity to arginine deprivation.

- vation *in vitro* is not determined by endogenous levels of arginine metabolic enzymes. *Cell Biol. Int.*, **2010**, 34(11), 1085-1089.
<http://dx.doi.org/10.1042/CBI20100451> PMID: 20653567
- [42] Han, R.Z.; Xu, G.C.; Dong, J.J.; Ni, Y. Arginine deiminase: Recent advances in discovery, crystal structure, and protein engineering for improved properties as an anti-tumor drug. *Appl. Microbiol. Biotechnol.*, **2016**, 100(11), 4747-4760.
<http://dx.doi.org/10.1007/s00253-016-7490-z> PMID: 27087524
- [43] Miyazaki, K.; Takaku, H.; Umeda, M.; Fujita, T.; Huang, W.D.; Kimura, T.; Yamashita, J.; Horio, T. Potent growth inhibition of human tumor cells in culture by arginine deiminase purified from a culture medium of a Mycoplasma-infected cell line. *Cancer Res.*, **1990**, 50(15), 4522-4527.
PMID: 2164440
- [44] Patil, M.D.; Bhaumik, J.; Babykutty, S.; Banerjee, U.C.; Fukumura, D. Arginine dependence of tumor cells: Targeting a chink in cancer's armor. *Oncogene*, **2016**, 35(38), 4957-4972.
<http://dx.doi.org/10.1038/onc.2016.37> PMID: 27109103
- [45] Holtsberg, F.W.; Ensor, C.M.; Steiner, M.R.; Bomalaski, J.S.; Clark, M.A. Poly(ethylene glycol) (PEG) conjugated arginine deiminase: Effects of PEG formulations on its pharmacological properties. *J. Control. Release*, **2002**, 80(1-3), 259-271.
[http://dx.doi.org/10.1016/S0168-3659\(02\)00042-1](http://dx.doi.org/10.1016/S0168-3659(02)00042-1) PMID: 11943403
- [46] Chow, A.K.; Yau, S.W.; Ng, L. Novel molecular targets in hepatocellular carcinoma. *World J. Clin. Oncol.*, **2020**, 11(8), 589-605.
<http://dx.doi.org/10.5306/wjco.v11.i8.589> PMID: 32879846
- [47] Fung, M.K.L.; Chan, G.C.F. Drug-induced amino acid deprivation as strategy for cancer therapy. *J. Hematol. Oncol.*, **2017**, 10(1), 144.
<http://dx.doi.org/10.1186/s13045-017-0509-9> PMID: 28750681
- [48] Dhankhar, R.; Gulati, P.; Kumar, S.; Kapoor, R.K. Arginine-lowering enzymes against cancer: A technocommercial analysis through patent landscape. *Expert Opin. Ther. Pat.*, **2018**, 28(8), 603-614.
<http://dx.doi.org/10.1080/13543776.2018.1508452> PMID: 30092168
- [49] Philip, R.; Campbell, E.; Wheatley, D.N. Arginine deprivation, growth inhibition and tumour cell death: 2. Enzymatic degradation of arginine in normal and malignant cell cultures. *Br. J. Cancer*, **2003**, 88(4), 613-623.
<http://dx.doi.org/10.1038/sj.bjc.6600681> PMID: 12592378
- [50] Wheatley, D.N.; Campbell, E. Arginine catabolism, liver extracts and cancer. *Pathol. Oncol. Res.*, **2002**, 8(1), 18-25.
<http://dx.doi.org/10.1007/BF03033696> PMID: 11994758
- [51] Li, L.H.; Wang, Y.; Chen, J.; Cheng, B.; Hu, J.H.; Zhou, Y.H.; Gao, X.; Gao, L.C.; Mei, X.F.; Sun, M.Y.; Zhang, Z.M.; Song, H.F. An Engineered Arginase FC Protein Inhibits Tumor Growth *in vitro* and *in vivo*. *Evidence-Based Complementary and Alternative Medicine*, **2013**.
- [52] Stone, E.M.; Glazer, E.S.; Chantranupong, L.; Cherukuri, P.; Breece, R.M.; Tierney, D.L.; Curley, S.A.; Iverson, B.L.; Georgiou, G. Replacing Mn(2+) with Co(2+) in human arginase i enhances cytotoxicity toward l-arginine auxotrophic cancer cell lines. *ACS Chem. Biol.*, **2010**, 5(3), 333-342.
<http://dx.doi.org/10.1021/cb900267j> PMID: 20050660
- [53] Yau, T.; Cheng, P.N.; Chan, P.; Chen, L.; Yuen, J.; Pang, R.; Fan, S.T.; Wheatley, D.N.; Poon, R.T. Preliminary efficacy, safety, pharmacokinetics, pharmacodynamics and quality of life study of pegylated recombinant human arginase 1 in patients with advanced hepatocellular carcinoma. *Invest. New Drugs*, **2015**, 33(2), 496-504.
<http://dx.doi.org/10.1007/s10637-014-0200-8> PMID: 25666409
- [54] Fultang, L.; Vardon, A.; De Santo, C.; Mussai, F. Molecular basis and current strategies of therapeutic arginine depletion for cancer. *Int. J. Cancer*, **2016**, 139(3), 501-509.
<http://dx.doi.org/10.1002/ijc.30051> PMID: 26913960
- [55] Chung, S.F.; Kim, C.F.; Tam, S.Y.; Choi, M.C.; So, P.K.; Wong, K.Y.; Leung, Y.C.; Lo, W.H. A bioengineered arginine-depleting enzyme as a long-lasting therapeutic agent against cancer. *Appl. Microbiol. Biotechnol.*, **2020**, 104(9), 3921-3934.
<http://dx.doi.org/10.1007/s00253-020-10484-4> PMID: 32144472
- [56] Schieber, M.; Chandel, N.S. ROS function in redox signaling and oxidative stress. *Curr. Biol.*, **2014**, 24(10), R453-R462.
<http://dx.doi.org/10.1016/j.cub.2014.03.034> PMID: 24845678
- [57] Hoffman, R.M. *Methionine Dependence of Cancer and Aging: Methods and protocols*; Humana Press: New York, NY, **2019**.
<http://dx.doi.org/10.1007/978-1-4939-8796-2>
- [58] Chaturvedi, S.; Hoffman, R.M.; Bertino, J.R. Exploiting methionine restriction for cancer treatment. *Biochem. Pharmacol.*, **2018**, 154, 170-173.
<http://dx.doi.org/10.1016/j.bcp.2018.05.003> PMID: 29733806
- [59] Cavuoto, P.; Fenech, M.F. A review of methionine dependency and the role of methionine restriction in cancer growth control and life-span extension. *Cancer Treat. Rev.*, **2012**, 38(6), 726-736.
<http://dx.doi.org/10.1016/j.ctrv.2012.01.004> PMID: 22342103
- [60] El-Sayed, A.S. Microbial L-methioninase: Production, molecular characterization, and therapeutic applications. *Appl. Microbiol. Biotechnol.*, **2010**, 86(2), 445-467.
<http://dx.doi.org/10.1007/s00253-009-2303-2> PMID: 20146062
- [61] Morozova, E.A.; Kulikova, V.V.; Yashin, D.V.; Anufrieva, N.V.; Anisimova, N.Y.; Revtovich, S.V.; Kotlov, M.I.; Belyi, Y.F.; Pokrovsky, V.S.; Demidkina, T.V. Kinetic Parameters and Cytotoxic Activity of Recombinant Methionine γ -Lyase from *Clostridium tetani*, *Clostridium sporogenes*, *Porphyromonas gingivalis* and *Citrobacter freundii*. *Acta Naturae*, **2013**, 5(3), 92-98.
<http://dx.doi.org/10.32607/20758251-2013-5-3-92-98> PMID: 24303205
- [62] Hoffman, R.M. Development of recombinant methioninase to target the general cancer-specific metabolic defect of methionine dependence: A 40-year odyssey. *Expert Opin. Biol. Ther.*, **2015**, 15(1), 21-31.
<http://dx.doi.org/10.1517/14712598.2015.963050> PMID: 25439528
- [63] Poirson-Bichat, F.; Gonçalves, R.A.; Miccoli, L.; Dutrillaux, B.; Poupon, M.F. Methionine depletion enhances the anti-tumoral efficacy of cytotoxic agents in drug-resistant human tumor xenografts. *Clin. Cancer Res.*, **2000**, 6(2), 643-653.
PMID: 10690550
- [64] Wang, Z.; Yip, L.Y.; Lee, J.H.J.; Wu, Z.; Chew, H.Y.; Chong, P.K.W.; Teo, C.C.; Ang, H.Y.; Peh, K.L.E.; Yuan, J.; Ma, S.; Choo, L.S.K.; Basri, N.; Jiang, X.; Yu, Q.; Hillmer, A.M.; Lim, W.T.; Lim, T.K.H.; Takano, A.; Tan, E.H.;

- Tan, D.S.W.; Ho, Y.S.; Lim, B.; Tam, W.L. Methionine is a metabolic dependency of tumor-initiating cells. *Nat. Med.*, **2019**, 25(5), 825-837.
<http://dx.doi.org/10.1038/s41591-019-0423-5> PMID: 31061538
- [65] Hu, J.; Cheung, N.K.V. Methionine depletion with recombinant methioninase: *in vitro* and *in vivo* efficacy against neuroblastoma and its synergism with chemotherapeutic drugs. *Int. J. Cancer*, **2009**, 124(7), 1700-1706.
<http://dx.doi.org/10.1002/ijc.24104> PMID: 19089915
- [66] Kokkinakis, D.M.; Hoffman, R.M.; Frenkel, E.P.; Wick, J.B.; Han, Q.; Xu, M.; Tan, Y.; Schold, S.C. Synergy between methionine stress and chemotherapy in the treatment of brain tumor xenografts in athymic mice. *Cancer Res.*, **2001**, 61(10), 4017-4023.
 PMID: 11358820
- [67] Lu, W.C.; Saha, A.; Yan, W.; Garrison, K.; Lamb, C.; Pandey, R.; Irani, S.; Lodi, A.; Lu, X.; Tiziani, S.; Zhang, Y.J.; Georgiou, G.; Stone, E. Enzyme-mediated depletion of serum l-Met abrogates prostate cancer growth *via* multiple mechanisms without evidence of systemic toxicity. *Proc. Natl. Acad. Sci. USA*, **2020**, 117(23), 13000-13011.
<http://dx.doi.org/10.1073/pnas.1917362117> PMID: 32434918
- [68] Sharma, B.; Singh, S.; Kanwar, S.S. L-Methionase: A Therapeutic Enzyme to Treat Malignancies. *Biomed Research International*, **2014**.
<http://dx.doi.org/10.1155/2014/506287>
- [69] Raboni, S.; Revtovich, S.; Demitri, N.; Giabbai, B.; Storici, P.; Cocconcetti, C.; Faggiano, S.; Rosini, E.; Pollegioni, L.; Galati, S.; Buschini, A.; Morozova, E.; Kulikova, V.; Nikulin, A.; Gabellieri, E.; Cioni, P.; Demidkina, T.; Mozzarelli, A. Engineering methionine γ -lyase from *Citrobacter freundii* for anticancer activity. *Biochim. Biophys. Acta. Proteins Proteomics*, **2018**, 1866(12), 1260-1270.
<http://dx.doi.org/10.1016/j.bbapap.2018.09.011> PMID: 30268810
- [70] Guillen, K.P.; Kurkjian, C.; Harrison, R.G. Targeted enzyme prodrug therapy for metastatic prostate cancer - a comparative study of L-methioninase, purine nucleoside phosphorylase, and cytosine deaminase. *J. Biomed. Sci.*, **2014**, 21, 65.
<http://dx.doi.org/10.1186/s12929-014-0065-3> PMID: 25047949
- [71] Van Rite, B.D.; Krais, J.J.; Cherry, M.; Sikavitsas, V.I.; Kurkjian, C.; Harrison, R.G. Antitumor activity of an enzyme prodrug therapy targeted to the breast tumor vasculature. *Cancer Invest.*, **2013**, 31(8), 505-510.
<http://dx.doi.org/10.3109/07357907.2013.840383> PMID: 24083814
- [72] Yang, Z.; Wang, J.; Lu, Q.; Xu, J.; Kobayashi, Y.; Takakura, T.; Takimoto, A.; Yoshioka, T.; Lian, C.; Chen, C.; Zhang, D.; Zhang, Y.; Li, S.; Sun, X.; Tan, Y.; Yagi, S.; Frenkel, E.P.; Hoffman, R.M. PEGylation confers greatly extended half-life and attenuated immunogenicity to recombinant methioninase in primates. *Cancer Res.*, **2004**, 64(18), 6673-6678.
<http://dx.doi.org/10.1158/0008-5472.CAN-04-1822> PMID: 15374983
- [73] Hoffman, R.M. PEG-methioninase. *Adv. Exp. Med. Biol.*, **2003**, 519, 69-79.
http://dx.doi.org/10.1007/0-306-47932-X_5 PMID: 12675209
- [74] Xin, L.; Caot, J.Q.; Liu, C.; Zeng, F.; Cheng, H.; Hu, X.Y.; Shao, J.H. Evaluation of rMETase-Loaded Stealth PLGA/Liposomes Modified with Anti-CAGE scFV for Treatment of Gastric Carcinoma. *J. Biomed. Nanotechnol.*, **2015**, 11(7), 1153-1161.
<http://dx.doi.org/10.1166/jbn.2015.2062> PMID: 26307838
- [75] Morozova, E.A.; Kulikova, V.V.; Faggiano, S.; Raboni, S.; Gabellieri, E.; Cioni, P.; Anufrieva, N.V.; Revtovich, S.V.; Demidkina, T.; Mozzarelli, A. Soluble and Nanoporous Silica Gel-Entrapped *C. freundii* Methionine γ -Lyase. *J. Nanosci. Nanotechnol.*, **2018**, 18(3), 2210-2219.
<http://dx.doi.org/10.1166/jnn.2018.14333> PMID: 29448748
- [76] Miki, K.; Al-Refai, W.; Xu, M.; Jiang, P.; Tan, Y.; Bouvet, M.; Zhao, M.; Gupta, A.; Chishima, T.; Shimada, H.; Makuuchi, M.; Moossa, A.R.; Hoffman, R.M. Methioninase gene therapy of human cancer cells is synergistic with recombinant methioninase treatment. *Cancer Res.*, **2000**, 60(10), 2696-2702.
 PMID: 10825143
- [77] Stone, E.; Paley, O.; Hu, J.; Ekerdt, B.; Cheung, N.K.; Georgiou, G. De novo engineering of a human cystathionine- γ -lyase for systemic (L)-Methionine depletion cancer therapy. *ACS Chem. Biol.*, **2012**, 7(11), 1822-1829.
<http://dx.doi.org/10.1021/cb300335j> PMID: 22963240
- [78] Gay, F.; Aguer, K.; Sénéchal, K.; Tainturier, A.; Berlier, W.; Maucourt-Boulch, D.; Honnorat, J.; Horand, F.; Godfrin, Y.; Bourgeaux, V. Methionine tumor starvation by erythrocyte-encapsulated methionine gamma-lyase activity controlled with per os vitamin B6. *Cancer Med.*, **2017**, 6(6), 1437-1452.
<http://dx.doi.org/10.1002/cam4.1086> PMID: 28544589
- [79] Bak, D.W.; Bechtel, T.J.; Falco, J.A.; Weerapana, E. Cysteine reactivity across the subcellular universe. *Curr. Opin. Chem. Biol.*, **2019**, 48, 96-105.
<http://dx.doi.org/10.1016/j.cbpa.2018.11.002> PMID: 30508703
- [80] Dixon, S.J.; Lemberg, K.M.; Lamprecht, M.R.; Skouta, R.; Zaitsev, E.M.; Gleason, C.E.; Patel, D.N.; Bauer, A.J.; Cantley, A.M.; Yang, W.S.; Morrison, B., III; Stockwell, B.R. Ferroptosis: An iron-dependent form of nonapoptotic cell death. *Cell*, **2012**, 149(5), 1060-1072.
<http://dx.doi.org/10.1016/j.cell.2012.03.042> PMID: 22632970
- [81] Cramer, S.L.; Saha, A.; Liu, J.; Tadi, S.; Tiziani, S.; Yan, W.; Triplett, K.; Lamb, C.; Alters, S.E.; Rowlinson, S.; Zhang, Y.J.; Keating, M.J.; Huang, P.; DiGiovanni, J.; Georgiou, G.; Stone, E. Systemic depletion of L-cyst(e)ine with cyst(e)inase increases reactive oxygen species and suppresses tumor growth. *Nat. Med.*, **2017**, 23(1), 120-127.
<http://dx.doi.org/10.1038/nm.4232> PMID: 27869804
- [82] Wang, W.; Green, M.; Choi, J.E.; Gijón, M.; Kennedy, P.D.; Johnson, J.K.; Liao, P.; Lang, X.; Kryczek, I.; Sell, A.; Xia, H.; Zhou, J.; Li, G.; Li, J.; Li, W.; Wei, S.; Vatan, L.; Zhang, H.; Szeliga, W.; Gu, W.; Liu, R.; Lawrence, T.S.; Lamb, C.; Tanno, Y.; Cieslik, M.; Stone, E.; Georgiou, G.; Chan, T.A.; Chinnaiyan, A.; Zou, W. CD8⁺ T cells regulate tumour ferroptosis during cancer immunotherapy. *Nature*, **2019**, 569(7755), 270-274.
<http://dx.doi.org/10.1038/s41586-019-1170-y> PMID: 31043744
- [83] Bonifacio, V.D.B.; Pereira, S.A.; Serpa, J.; Vicente, J.B. Cysteine metabolic circuitries: Druggable targets in cancer. *Br. J. Cancer*, **2020**.
 PMID: 33223534
- [84] Kshattri, S.; Saha, A.; Gries, P.; Tiziani, S.; Stone, E.; Georgiou, G.; DiGiovanni, J. Enzyme-mediated depletion of L-cyst(e)ine synergizes with thioredoxin reductase inhibition

- for suppression of pancreatic tumor growth. *NPJ Precis Oncol*, **2019**, 3, 16.
<http://dx.doi.org/10.1038/s41698-019-0088-z> PMID: 31231686
- [85] Jones, C.L.; Stevens, B.M.; D'Alessandro, A.; Culp-Hill, R.; Reisz, J.A.; Pei, S.; Gustafson, A.; Khan, N.; DeGregori, J.; Pollyea, D.A.; Jordan, C.T. Cysteine depletion targets leukemia stem cells through inhibition of electron transport complex II. *Blood*, **2019**, 134(4), 389-394.
<http://dx.doi.org/10.1182/blood.2019898114> PMID: 31101624
- [86] Graham, N.A.; Tahmasian, M.; Kohli, B.; Komisopoulou, E.; Zhu, M.; Vivanco, I.; Teitell, M.A.; Wu, H.; Ribas, A.; Lo, R.S.; Mellingerhoff, I.K.; Mischel, P.S.; Graeber, T.G. Glucose deprivation activates a metabolic and signaling amplification loop leading to cell death. *Mol. Syst. Biol.*, **2012**, 8, 589.
<http://dx.doi.org/10.1038/msb.2012.20> PMID: 22735335
- [87] Liu, Y.; Nan, X.; Shi, W.; Liu, X.; He, Z.; Sun, Y.N.; Ge, D.T. A glucose biosensor based on the immobilization of glucose oxidase and Au nanocomposites with polynorepinephrine. *RSC Advances*, **2019**, 9(29), 16439-16446.
<http://dx.doi.org/10.1039/C9RA02054C>
- [88] Yu, S.; Chen, Z.; Zeng, X.; Chen, X.; Gu, Z. Advances in nanomedicine for cancer starvation therapy. *Theranostics*, **2019**, 9(26), 8026-8047.
<http://dx.doi.org/10.7150/thno.38261> PMID: 31754379
- [89] Wang, C.H.; Yang, J.X.; Dong, C.Y.; Shi, S. Glucose Oxidase-Related Cancer Therapies. *Adv. Ther.*, **2020**, 3(10)
- [90] Bankar, S.B.; Bule, M.V.; Singhal, R.S.; Ananthanarayan, L. Glucose oxidase--an overview. *Biotechnol. Adv.*, **2009**, 27(4), 489-501.
<http://dx.doi.org/10.1016/j.biotechadv.2009.04.003> PMID: 19374943
- [91] Dinda, S.; Sarkar, S.; Das, P.K. Glucose oxidase mediated targeted cancer-starving therapy by biotinylated self-assembled vesicles. *Chem. Commun. (Camb.)*, **2018**, 54(71), 9929-9932.
<http://dx.doi.org/10.1039/C8CC03599G> PMID: 30116805
- [92] Wang, M.; Wang, D.; Chen, Q.; Li, C.; Li, Z.; Lin, J. Recent Advances in Glucose-Oxidase-Based Nanocomposites for Tumor Therapy. *Small*, **2019**, 15(51), e1903895.
<http://dx.doi.org/10.1002/smll.201903895> PMID: 31747128
- [93] Zhang, Y.H.; Qiu, W.X.; Zhang, M.; Zhang, L.; Zhang, X.Z. MnO₂ Motor: A Prospective Cancer-Starving Therapy Promoter. *ACS Appl. Mater. Interfaces*, **2018**, 10(17), 15030-15039.
<http://dx.doi.org/10.1021/acsami.8b01818> PMID: 29633614
- [94] Sun, D.; Qi, G.; Ma, K.; Qu, X.; Xu, W.; Xu, S.; Jin, Y. Tumor Microenvironment- Activated Degradable Multifunctional Nanoreactor for Synergistic Cancer Therapy and Glucose SERS Feedback. *iScience*, **2020**, 23(7), 101274.
- [95] Pastan, I.; Hassan, R.; Fitzgerald, D.J.; Kreitman, R.J. Immunotoxin therapy of cancer. *Nat. Rev. Cancer*, **2006**, 6(7), 559-565.
<http://dx.doi.org/10.1038/nrc1891> PMID: 16794638
- [96] Pastan, I.; Hassan, R.; FitzGerald, D.J.; Kreitman, R.J. Immunotoxin treatment of cancer. *Annu. Rev. Med.*, **2007**, 58, 221-237.
<http://dx.doi.org/10.1146/annurev.med.58.070605.115320> PMID: 17059365
- [97] Akbari, B.; Farajnia, S.; Ahdi Khosroshahi, S.; Safari, F.; Yousefi, M.; Dariushejad, H.; Rahbarnia, L. Immunotoxins in cancer therapy: Review and update. *Int. Rev. Immunol.*, **2017**, 36(4), 207-219.
<http://dx.doi.org/10.1080/08830185.2017.1284211> PMID: 28282218
- [98] Allahyari, H.; Heidari, S.; Ghamgosha, M.; Saffarian, P.; Amani, J. Immunotoxin: A new tool for cancer therapy. *Tumour Biol.*, **2017**, 39(2), 1010428317692226.
<http://dx.doi.org/10.1177/1010428317692226> PMID: 28218037
- [99] Wong, J.H.; Bao, H.; Ng, T.B.; Chan, H.H.L.; Ng, C.C.W.; Man, G.C.W.; Wang, H.; Guan, S.; Zhao, S.; Fang, E.F.; Rolka, K.; Liu, Q.; Li, C.; Sha, O.; Xia, L. New ribosome-inactivating proteins and other proteins with protein synthesis-inhibiting activities. *Appl. Microbiol. Biotechnol.*, **2020**, 104(10), 4211-4226.
<http://dx.doi.org/10.1007/s00253-020-10457-7> PMID: 32193575
- [100] Rust, A.; Partridge, L.J.; Davletov, B.; Hautbergue, G.M. The Use of Plant-Derived Ribosome Inactivating Proteins in Immunotoxin Development: Past, Present and Future Generations. *Toxins (Basel)*, **2017**, 9(11), 9.
<http://dx.doi.org/10.3390/toxins9110344> PMID: 29076988
- [101] Blakey, D.C.; Watson, G.J.; Knowles, P.P.; Thorpe, P.E. Effect of chemical deglycosylation of ricin A chain on the *in vivo* fate and cytotoxic activity of an immunotoxin composed of ricin A chain and anti-Thy 1.1 antibody. *Cancer Res.*, **1987**, 47(4), 947-952.
 PMID: 3492271
- [102] Lu, J.-Q.; Zhu, Z.-N.; Zheng, Y.-T.; Shaw, P.-C. Engineering of Ribosome-inactivating Proteins for Improving Pharmacological Properties. *Toxins (Basel)*, **2020**, 12(3), 12.
<http://dx.doi.org/10.3390/toxins12030167> PMID: 32182799
- [103] Cizeau, J.; Grenkow, D.M.; Brown, J.G.; Entwistle, J.; MacDonald, G.C. Engineering and biological characterization of VB6-845, an anti-EpCAM immunotoxin containing a T-cell epitope-depleted variant of the plant toxin bouganin. *J. Immunother.*, **2009**, 32(6), 574-584.
<http://dx.doi.org/10.1097/CJI.0b013e3181a6981c> PMID: 19483652
- [104] Mazar, R.; Eberle, J.A.; Hu, X.; Vassall, A.N.; Onda, M.; Beers, R.; Lee, E.C.; Kreitman, R.J.; Lee, B.; Baker, D.; King, C.; Hassan, R.; Benhar, I.; Pastan, I. Recombinant immunotoxin for cancer treatment with low immunogenicity by identification and silencing of human T-cell epitopes. *Proceedings of the National Academy of Sciences*, **2014**, p. 111.
<http://dx.doi.org/10.1073/pnas.1405153111>
- [105] Cheung, L.S.; Fu, J.; Kumar, P.; Kumar, A.; Urbanowski, M.E.; Ihms, E.A.; Parveen, S.; Bullen, C.K.; Patrick, G.J.; Harrison, R.; Murphy, J.R.; Pardoll, D.M.; Bishai, W.R. Second-generation IL-2 receptor-targeted diphtheria fusion toxin exhibits antitumor activity and synergy with anti-PD-1 in melanoma. *Proceedings of the National Academy of Sciences*, **2019**, p. 116.
<http://dx.doi.org/10.1073/pnas.1815087116>
- [106] Zuppone, S.; Fabbrini, M.S.; Vago, R. Hosts for Hostile Protein Production: The Challenge of Recombinant Immunotoxin Expression. *Biomedicines*, **2019**, 7(2), 7.
<http://dx.doi.org/10.3390/biomedicines7020038> PMID: 31108917
- [107] Olsen, E.; Duvic, M.; Frankel, A.; Kim, Y.; Martin, A.; Vonderheid, E.; Jegasothy, B.; Wood, G.; Gordon, M.; Heald, P.; Oseroff, A.; Pinter-Brown, L.; Bowen, G.; Kuzel, T.; Fivenson, D.; Foss, F.; Glode, M.; Molina, A.; Knobler, E.; Stewart, S.; Cooper, K.; Stevens, S.; Craig, F.; Reuben, J.; Bacha, P.; Nichols, J. Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma. *J. Clin. Oncol.*, **2001**, 19(2), 376-388.

- <http://dx.doi.org/10.1200/JCO.2001.19.2.376> PMID: 11208829
- [108] Jen, E.Y.; Gao, X.; Li, L.; Zhuang, L.; Simpson, N.E.; Aryal, B.; Wang, R.; Przepiorka, D.; Shen, Y.L.; Leong, R.; Liu, C.; Sheth, C.M.; Bowen, S.; Goldberg, K.B.; Farrell, A.T.; Blumenthal, G.M.; Pazdur, R. FDA Approval Summary: Tagraxofusp-erzs For Treatment of Blastic Plasmacytoid Dendritic Cell Neoplasm. *Clin. Cancer Res.*, **2020**, *26*(3), 532-536.
<http://dx.doi.org/10.1158/1078-0432.CCR-19-2329> PMID: 31548341
- [109] Kreitman, R.J.; Dearden, C.; Zinzani, P.L.; Delgado, J.; Karlin, L.; Robak, T.; Gladstone, D.E.; le Coutre, P.; Dietrich, S.; Gotic, M.; Larratt, L.; Offner, F.; Schiller, G.; Swords, R.; Bacon, L.; Bocchia, M.; Bouabdallah, K.; Breems, D.A.; Cortelezzi, A.; Dinner, S.; Doubek, M.; Gjertsen, B.T.; Gobbi, M.; Hellmann, A.; Lepretre, S.; Maloïsel, F.; Ravandi, F.; Rousselot, P.; Rummel, M.; Sidiqi, T.; Tadmor, T.; Troussard, X.; Yi, C.A.; Saglio, G.; Roboz, G.J.; Balic, K.; Standifer, N.; He, P.; Marshall, S.; Wilson, W.; Pastan, I.; Yao, N.-S.; Giles, F. Moxetumomab pasudotox in relapsed/refractory hairy cell leukemia. *Leukemia*, **2018**, *32*(8), 1768-1777.
<http://dx.doi.org/10.1038/s41375-018-0210-1> PMID: 30030507
- [110] Mazor, R.; King, E.M.; Onda, M.; Cuburu, N.; Addissie, S.; Crown, D.; Liu, X.-F.; Kishimoto, T.K.; Pastan, I. Tolerogenic nanoparticles restore the antitumor activity of recombinant immunotoxins by mitigating immunogenicity. *Proceedings of the National Academy of Sciences*, **2018**, p. 115.
<http://dx.doi.org/10.1073/pnas.1717063115>
- [111] Tang, Y.; Liang, J.; Wu, A.; Chen, Y.; Zhao, P.; Lin, T.; Zhang, M.; Xu, Q.; Wang, J.; Huang, Y. Co-Delivery of Trichosanthin and Albendazole by Nano-Self-Assembly for Overcoming Tumor Multidrug-Resistance and Metastasis. *ACS Appl. Mater. Interfaces*, **2017**, *9*(32), 26648-26664.
<http://dx.doi.org/10.1021/acsami.7b05292> PMID: 28741923
- [112] Kim, J.-S.; Jun, S.-Y.; Kim, Y.-S. Critical Issues in the Development of Immunotoxins for Anticancer Therapy. *J. Pharm. Sci.*, **2020**, *109*(1), 104-115.
<http://dx.doi.org/10.1016/j.xphs.2019.10.037> PMID: 31669121
- [113] Gotte, G.; Menegazzi, M. Biological Activities of Secretory RNases: Focus on Their Oligomerization to Design Antitumor Drugs. *Front. Immunol.*, **2019**, *10*, 2626.
<http://dx.doi.org/10.3389/fimmu.2019.02626> PMID: 31849926
- [114] De Lorenz, C.; D'Alessio, G. From immunotoxins to immunoRNases. *Curr. Pharm. Biotechnol.*, **2008**, *9*(3), 210-214.
<http://dx.doi.org/10.2174/138920108784567254> PMID: 18673286
- [115] Schirrmann, T.; Krauss, J.; Arndt, M.A.; Rybak, S.M.; Dübel, S. Targeted therapeutic RNases (ImmunoRNases). *Expert Opin. Biol. Ther.*, **2009**, *9*(1), 79-95.
<http://dx.doi.org/10.1517/14712590802631862> PMID: 19063695
- [116] Jordaan, S.; Akinrinmade, O.A.; Nachreiner, T.; Cremer, C.; Naran, K.; Chetty, S.; Barth, S. Updates in the Development of ImmunoRNases for the Selective Killing of Tumor Cells. *Biomedicine*, **2018**, *6*(1), 6.
<http://dx.doi.org/10.3390/biomedicine6010028> PMID: 29510557
- [117] Lee, J.E.; Raines, R.T. Ribonucleases as novel chemotherapeutics : The ranpirinase example. *BioDrugs*, **2008**, *22*(1), 53-58.
<http://dx.doi.org/10.2165/00063030-200822010-00006> PMID: 18215091
- [118] Costanzi, J.; Sidransky, D.; Navon, A.; Goldsweig, H. Ribonucleases as a novel pro-apoptotic anticancer strategy: Review of the preclinical and clinical data for ranpirinase. *Cancer Invest.*, **2005**, *23*(7), 643-650.
<http://dx.doi.org/10.1080/07357900500283143> PMID: 16305992
- [119] SS, K.; R, K. Ribonuclease as anticancer therapeutics. *Enzyme Engineering*, **2017**, *06*
- [120] Squiquera, L.; Taxman, D.J.; Brendle, S.A.; Torres, R.; Sulley, J.; Hodge, T.; Christensen, N.; Sidransky, D. Ranpirinase eradicates human papillomavirus in cultured cells and heals anogenital warts in a Phase I study. *Antivir. Ther.*, **2017**, *22*(3), 247-255.
<http://dx.doi.org/10.3851/IMP3133> PMID: 28121292
- [121] Saxena, S.K.; Gravell, M.; Wu, Y.-N.; Mikulski, S.M.; Shogen, K.; Ardelt, W.; Youle, R.J. Inhibition of HIV-1 production and selective degradation of viral RNA by an amphibian ribonuclease. *J. Biol. Chem.*, **1996**, *271*(34), 20783-20788.
<http://dx.doi.org/10.1074/jbc.271.34.20783> PMID: 8702832
- [122] Brand, R.M.; Siegel, A.; Myerski, A.; Metter, E.J.; Engstrom, J.; Brand, R.E.; Squiquera, L.; Hodge, T.; Sulley, J.; Cranston, R.D.; McGowan, I. Ranpirinase Reduces HIV-1 Infection and Associated Inflammatory Changes in a Human Colorectal Explant Model. *AIDS Res. Hum. Retroviruses*, **2018**, *34*(10), 838-848.
<http://dx.doi.org/10.1089/aid.2017.0308> PMID: 29936861
- [123] Hodge, T.; Draper, K.; Brasel, T.; Freiberg, A.; Squiquera, L.; Sidransky, D.; Sulley, J.; Taxman, D.J. Antiviral effect of ranpirinase against Ebola virus. *Antiviral Res.*, **2016**, *132*, 210-218.
<http://dx.doi.org/10.1016/j.antiviral.2016.06.009> PMID: 27350309
- [124] Hauge, A.; Rofstad, E.K. Antifibrotic therapy to normalize the tumor microenvironment. *J. Transl. Med.*, **2020**, *18*(1), 207.
<http://dx.doi.org/10.1186/s12967-020-02376-y> PMID: 32434573
- [125] Kultti, A.; Li, X.; Jiang, P.; Thompson, C.B.; Frost, G.I.; Shepard, H.M. Therapeutic targeting of hyaluronan in the tumor stroma. *Cancers (Basel)*, **2012**, *4*(3), 873-903.
<http://dx.doi.org/10.3390/cancers4030873> PMID: 24213471
- [126] Auvinen, P.; Tammi, R.; Parkkinen, J.; Tammi, M.; Ågren, U.; Johansson, R.; Hirvikoski, P.; Eskelinen, M.; Kosma, V.-M. Hyaluronan in Peritumoral Stroma and Malignant Cells Associates with Breast Cancer Spreading and Predicts Survival. *Am. J. Pathol.*, **2000**, *156*.
- [127] Theocharis, A.D.; Skandalis, S.S.; Tzanakakis, G.N.; Karamanos, N.K. Proteoglycans in health and disease: Novel roles for proteoglycans in malignancy and their pharmacological targeting. *FEBS J.*, **2010**, *277*(19), 3904-3923.
<http://dx.doi.org/10.1111/j.1742-4658.2010.07800.x> PMID: 20840587
- [128] Provenzano, P.P.; Cuevas, C.; Chang, A.E.; Goel, V.K.; Von Hoff, D.D.; Hingorani, S.R. Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma. *Cancer Cell*, **2012**, *21*.
- [129] Grass, G.D.; Dai, L.; Qin, Z.; Parsons, C.; Toole, B.P. CD147: Regulator of hyaluronan signaling in invasiveness and chemoresistance. *Adv. Cancer Res.*, **2014**, *123*, 351-373.
<http://dx.doi.org/10.1016/B978-0-12-800092-2.00013-7> PMID: 25081536
- [130] Shepard, H.M. Breaching the Castle Walls: Hyaluronan Depletion as a Therapeutic Approach to Cancer Therapy. *Front. Oncol.*, **2015**, *5*, 192.

- <http://dx.doi.org/10.3389/fonc.2015.00192> PMID: 26380222
- [131] de Lemos, M.L.; Dar Santos, A. Clinical considerations of hyaluronidase as an adjunct to subcutaneous rituximab injection. *J. Oncol. Pharm. Pract.*, **2019**, *25*(4), 964-965. <http://dx.doi.org/10.1177/1078155218817819> PMID: 30975068
- [132] Duco, M.R.; Murdock, J.L.; Reeves, D.J. Trastuzumab/Hyaluronidase-oysk: A New Option for Patients With HER2-Positive Breast Cancer. *Ann. Pharmacother.*, **2020**, *54*(3), 254-261. <http://dx.doi.org/10.1177/1060028019877936> PMID: 31595774
- [133] Heo, Y.-A.; Syed, Y.Y. Subcutaneous Trastuzumab: A Review in HER2-Positive Breast Cancer. *Target. Oncol.*, **2019**, *14*(6), 749-758. <http://dx.doi.org/10.1007/s11523-019-00684-y> PMID: 31686307
- [134] Gao, J.J.; Osgood, C.L.; Gong, Y.; Zhang, H.; Bloomquist, E.W.; Jiang, X.; Qiu, J.; Yu, J.; Song, P.; Rahman, A.; Chiu, H.-J.; Ricks, T.K.; Rizvi, F.; Hou, S.; Wilson, W.; Abukhdeir, A.M.; Seidman, J.; Ghosh, S.; Philip, R.; Pierce, W.F.; Bhatnagar, V.; Kluetz, P.G.; Pazdur, R.; Beaver, J.A.; Amiri-Kordestani, L. FDA Approval Summary: Pertuzumab, Trastuzumab, and Hyaluronidase-zzxf injection for subcutaneous use in Patients with HER2-Positive Breast Cancer. *Clin. Cancer Res.*, **2020**. <http://dx.doi.org/10.1158/1078-0432.CCR-20-3474> PMID: 33188141
- [135] Lamb, Y.N. Daratumumab: A Review in Combination Therapy for Transplant-Eligible Newly Diagnosed Multiple Myeloma. *Drugs*, **2020**, *80*(14), 1455-1464. <http://dx.doi.org/10.1007/s40265-020-01385-x> PMID: 32936436
- [136] Maneval, D.C.; Caster, L.; Derunes, C.; Locke, K.W.; Muhsin, M.; Sauter, S.; Sekulovich, R.E.; Thompson, C.B.; LaBarre, M.J. Pegvorhyaluronidase alfa: A PEGylated recombinant human hyaluronidase PH20 for the treatment of cancers that accumulate hyaluronan; Polymer-Protein Conjugates, **2020**, pp. 175-204.
- [137] Infante, J.R.; Korn, R.L.; Rosen, L.S.; LoRusso, P.; Dychter, S.S.; Zhu, J.; Maneval, D.C.; Jiang, P.; Shepard, H.M.; Frost, G.; Von Hoff, D.D.; Borad, M.J.; Ramanathan, R.K. Phase 1 trials of PEGylated recombinant human hyaluronidase PH20 in patients with advanced solid tumours. *Br. J. Cancer*, **2018**, *118*.
- [138] Hingorani, S.R.; Zheng, L.; Bullock, A.J.; Seery, T.E.; Harris, W.P.; Sigal, D.S.; Braiteh, F.; Ritch, P.S.; Zalupski, M.M.; Bahary, N.; Oberstein, P.E.; Wang-Gillam, A.; Wu, W.; Chondros, D.; Jiang, P.; Khelifa, S.; Pu, J.; Aldrich, C.; Hendifar, A.E. HALO 202: Randomized Phase II Study of PEGPH20 Plus Nab-Paclitaxel/Gemcitabine Versus Nab-Paclitaxel/Gemcitabine in Patients With Untreated, Metastatic Pancreatic Ductal Adenocarcinoma. *J. Clin. Oncol.*, **2018**, *36*(4), 359-366. <http://dx.doi.org/10.1200/JCO.2017.74.9564> PMID: 29232172
- [139] Doherty, G.J.; Tempero, M.; Corrie, P.G. HALO-109-301: A Phase III trial of PEGPH20 (with gemcitabine and nab-paclitaxel) in hyaluronic acid-high stage IV pancreatic cancer. *Future Oncol.*, **2018**, *14*(1), 13-22. <http://dx.doi.org/10.2217/fon-2017-0338> PMID: 29235360
- [140] Wong, K.M.; Horton, K.J.; Coveler, A.L.; Hingorani, S.R.; Harris, W.P. Targeting the Tumor Stroma: The Biology and Clinical Development of Pegylated Recombinant Human Hyaluronidase (PEGPH20). *Curr. Oncol. Rep.*, **2017**, *19*(7), 47. <http://dx.doi.org/10.1007/s11912-017-0608-3> PMID: 28589527
- [141] Soundararajan, R.; Wang, G.; Petkova, A.; Uchegbu, I.F.; Schätzlein, A.G. Hyaluronidase Coated Molecular Envelope Technology Nanoparticles Enhance Drug Absorption via the Subcutaneous Route. *Mol. Pharm.*, **2020**, *17*(7), 2599-2611. <http://dx.doi.org/10.1021/acs.molpharmaceut.0c00294> PMID: 32379457
- [142] Scodeller, P.; Catalano, P.N.; Salguero, N.; Duran, H.; Wolosiuk, A.; Soler-Illia, G.J.A.A. Hyaluronan degrading silica nanoparticles for skin cancer therapy. *Nanoscale*, **2013**, *5*(20), 9690-9698. <http://dx.doi.org/10.1039/c3nr02787b> PMID: 23969526
- [143] Zhou, H.; Fan, Z.; Deng, J.; Lemons, P.K.; Arhontoulis, D.C.; Bowne, W.B.; Cheng, H. Hyaluronidase Embedded in Nanocarrier PEG Shell for Enhanced Tumor Penetration and Highly Efficient Antitumor Efficacy. *Nano Lett.*, **2016**, *16*(5), 3268-3277. <http://dx.doi.org/10.1021/acs.nanolett.6b00820> PMID: 27057591
- [144] Zhou, H.; Fan, Z.; Lemons, P.K.; Cheng, H. A facile approach to functionalize cell membrane-coated nanoparticles. *Theranostics*, **2016**, *6*(7), 1012-1022. <http://dx.doi.org/10.7150/thno.15095> PMID: 27217834
- [145] Kim, S.S.; Kim, H.K.; Kim, H.; Lee, W.T.; Lee, E.S.; Oh, K.T.; Choi, H.-G.; Youn, Y.S. Hyperthermal paclitaxel-bound albumin nanoparticles co-loaded with indocyanine green and hyaluronidase for treating pancreatic cancers. *Arch. Pharm. Res.*, **2020**. <http://dx.doi.org/10.1007/s12272-020-01264-9> PMID: 32803685
- [146] Dai, J.; Han, S.; Ju, F.; Han, M.; Xu, L.; Zhang, R.; Sun, Y. Preparation and evaluation of tumour microenvironment response multistage nanoparticles for epirubicin delivery and deep tumour penetration. *Artif. Cells Nanomed. Biotechnol.*, **2018**, *46*(sup2), 860-873. <http://dx.doi.org/10.1080/21691401.2018.1470528> PMID: 29771149
- [147] Yeldandi, A.V.; Yeldandi, V.; Kumar, S.; Murthy, C.V.; Wang, X.D.; Alvares, K.; Rao, M.S.; Reddy, J.K. Molecular evolution of the urate oxidase-encoding gene in hominoid primates: Nonsense mutations. *Gene*, **1991**, *109*(2), 281-284. [http://dx.doi.org/10.1016/0378-1119\(91\)90622-I](http://dx.doi.org/10.1016/0378-1119(91)90622-I) PMID: 1765273
- [148] Alakel, N.; Middeke, J.M.; Schetelig, J.; Bornhäuser, M. Prevention and treatment of tumor lysis syndrome, and the efficacy and role of rasburicase. *OncoTargets Ther.*, **2017**, *10*, 597-605. <http://dx.doi.org/10.2147/OTT.S103864> PMID: 28203093
- [149] Bayol, A.; Capdevielle, J.; Malazzi, P.; Buzy, A.; Claude Bonnet, M.; Colloc'h, N.; Momon, J.-P.; Loyaux, D.; Ferrara, P. Modification of a reactive cysteine explains differences between rasburicase and Uricozyme, a natural *Aspergillus flavus* uricase. *Biotechnol. Appl. Biochem.*, **2002**, *36*(1), 21-31. <http://dx.doi.org/10.1042/BA20010083> PMID: 12149119
- [150] Owens, R.E.; Swanson, H.; Twilla, J.D. Hemolytic Anemia Induced by Pegloticase Infusion in a Patient With G6PD Deficiency. *JCR: Journal of Clinical Rheumatology*, **2016**, *22*
- [151] Pui, C.-H.; Mahmoud, H.H.; Wiley, J.M.; Woods, G.M.; Leverger, G.; Camitta, B.; Hastings, C.; Blaney, S.M.; Relling, M.V.; Reaman, G.H. Recombinant urate oxidase for the prophylaxis or treatment of hyperuricemia in patients With leukemia or lymphoma. *J. Clin. Oncol.*, **2001**, *19*(3), 697-704.

- <http://dx.doi.org/10.1200/JCO.2001.19.3.697> PMID: 11157020
- [152] Sherman, M.R.; Saifer, M.G.P.; Perez-Ruiz, F. PEG-uricase in the management of treatment-resistant gout and hyperuricemia. *Adv. Drug Deliv. Rev.*, **2008**, *60*(1), 59-68. <http://dx.doi.org/10.1016/j.addr.2007.06.011> PMID: 17826865
- [153] Hershfield, M.S.; Roberts, L.J.; Ganson, N.J.; Kelly, S.J.; Santisteban, I.; Scarlett, E.; Jagers, D.; Sundry, J.S. Treating gout with pegloticase, a PEGylated urate oxidase, provides insight into the importance of uric acid as an antioxidant *in vivo*. *Proceedings of the National Academy of Sciences*, **2010**, p. 107. <http://dx.doi.org/10.1073/pnas.1001072107>
- [154] Schlesinger, N.; Lipsky, P.E. Pegloticase treatment of chronic refractory gout: Update on efficacy and safety. *Semin. Arthritis Rheum.*, **2020**, *50*(3S), S31-S38. <http://dx.doi.org/10.1016/j.semarthrit.2020.04.011> PMID: 32620200
- [155] Najjari, A.; Rahimi, H.; Nojoumi, S.A.; Omidinia, E. Computational Approach for Rational Design of Fusion Uricase with PAS Sequences. *Int. J. Mol. Cell. Med.*, **2020**, *9*(1), 90-103. PMID: 32832488
- [156] Geraths, C.; Daoud-El Baba, M.; Charpin-El Hamri, G.; Weber, W. A biohybrid hydrogel for the urate-responsive release of urate oxidase. *J. Control. Release*, **2013**, *171*(1), 57-62. <http://dx.doi.org/10.1016/j.jconrel.2013.06.037> PMID: 23838153
- [157] Xiong, H.; Zhou, Y.; Zhou, Q.; He, D.; Wan, S.; Tan, Q.; Zhang, M.; Deng, X.; Zhang, J. Nanosomal Microassemblies for Highly Efficient and Safe Delivery of Therapeutic Enzymes. *ACS Appl. Mater. Interfaces*, **2015**, *7*(36), 20255-20263. <http://dx.doi.org/10.1021/acsami.5b05758> PMID: 26325262
- [158] Yoshimoto, M.; Takaki, N.; Yamasaki, M. Catalase-conjugated liposomes encapsulating glucose oxidase for controlled oxidation of glucose with decomposition of hydrogen peroxide produced. *Colloids Surf. B Biointerfaces*, **2010**, *79*(2), 403-408. <http://dx.doi.org/10.1016/j.colsurfb.2010.05.006> PMID: 20537512
- [159] Zhou, Y.; Zhang, M.; He, D.; Hu, X.; Xiong, H.; Wu, J.; Zhu, B.; Zhang, J. Uricase alkaline enzymosomes with enhanced stabilities and anti-hyperuricemia effects induced by favorable microenvironmental changes. *Sci. Rep.*, **2016**, *7*, 20136. <http://dx.doi.org/10.1038/srep20136> PMID: 26823332
- [160] Jung, S.; Kwon, I. Synergistic Degradation of a Hyperuricemia-Causing Metabolite Using One-Pot Enzyme-Nanozyme Cascade Reactions. *Sci. Rep.*, **2017**, *7*, 44330. <http://dx.doi.org/10.1038/srep44330> PMID: 28287162
- [161] Kim, S.; Kim, M.; Jung, S.; Kwon, K.; Park, J.; Kim, S.; Kwon, I.; Tae, G. Co-delivery of therapeutic protein and catalase-mimic nanoparticle using a biocompatible nanocarrier for enhanced therapeutic effect. *J. Control. Release*, **2019**, *309*, 181-189. <http://dx.doi.org/10.1016/j.jconrel.2019.07.038> PMID: 31356840
- [162] Minton, N.P.; Atkinson, T.; Sherwood, R.F. Molecular cloning of the *Pseudomonas* carboxypeptidase G2 gene and its expression in *Escherichia coli* and *Pseudomonas putida*. *J. Bacteriol.*, **1983**, *156*(3), 1222-1227. <http://dx.doi.org/10.1128/jb.156.3.1222-1227.1983> PMID: 6358192
- [163] McCullough, J.L.; Chabner, B.A.; Bertino, J.R. Purification and properties of carboxypeptidase G 1. *J. Biol. Chem.*, **1971**, *246*(23), 7207-7213. [http://dx.doi.org/10.1016/S0021-9258\(19\)45873-0](http://dx.doi.org/10.1016/S0021-9258(19)45873-0) PMID: 5129727
- [164] Sherwood, R.F.; Melton, R.G.; Alwan, S.M.; Hughes, P. Purification and properties of carboxypeptidase G2 from *Pseudomonas* sp. strain RS-16. Use of a novel triazine dye affinity method. *Eur. J. Biochem.*, **1985**, *148*(3), 447-453. <http://dx.doi.org/10.1111/j.1432-1033.1985.tb08860.x> PMID: 3838935
- [165] Rowsell, S.; Paupit, R.A.; Tucker, A.D.; Melton, R.G.; Blow, D.M.; Brick, P. Crystal structure of carboxypeptidase G2, a bacterial enzyme with applications in cancer therapy. *Structure*, **1997**, *5*(3), 337-347. [http://dx.doi.org/10.1016/S0969-2126\(97\)00191-3](http://dx.doi.org/10.1016/S0969-2126(97)00191-3) PMID: 9083113
- [166] Green, J.M. Glucarpidase to combat toxic levels of methotrexate in patients. *Ther. Clin. Risk Manag.*, **2012**, *8*, 403-413. <http://dx.doi.org/10.2147/TCRM.S30135> PMID: 23209370
- [167] Al-Qahtani, A.D.; Bashraheel, S.S.; Rashidi, F.B.; O'Connor, C.D.; Romero, A.R.; Domling, A.; Goda, S.K. Production of "biobetter" variants of glucarpidase with enhanced enzyme activity. *Biomed. Pharmacother.*, **2019**, *112*(108725), 108725. <http://dx.doi.org/10.1016/j.biopha.2019.108725> PMID: 30970523
- [168] Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2012/125327lbl.pdf
- [169] Bedoui, Y.; Guillot, X.; Sélambarom, J.; Guiraud, P.; Giry, C.; Jaffar-Bandjee, M.C.; Ralandison, S.; Gasque, P. Methotrexate an Old Drug with New Tricks. *Int. J. Mol. Sci.*, **2019**, *20*(20), E5023. <http://dx.doi.org/10.3390/ijms20205023> PMID: 31658782
- [170] Widemann, B.C.; Adamson, P.C. Understanding and managing methotrexate nephrotoxicity. *Oncologist*, **2006**, *11*(6), 694-703. <http://dx.doi.org/10.1634/theoncologist.11-6-694> PMID: 16794248
- [171] Hansen, H.H.; Selawry, O.S.; Holland, J.F.; McCall, C.B. The variability of individual tolerance to methotrexate in cancer patients. *Br. J. Cancer*, **1971**, *25*(2), 298-305. <http://dx.doi.org/10.1038/bjc.1971.38> PMID: 4256007
- [172] Buchen, S.; Ngampolo, D.; Melton, R.G.; Hasan, C.; Zoubek, A.; Henze, G.; Bode, U.; Fleischhack, G. Carboxypeptidase G2 rescue in patients with methotrexate intoxication and renal failure. *Br. J. Cancer*, **2005**, *92*(3), 480-487. <http://dx.doi.org/10.1038/sj.bjc.6602337> PMID: 15668713
- [173] Tuffaha, H.W.; Al Omar, S. Glucarpidase for the treatment of life-threatening methotrexate overdose. *Drugs Today (Barc)*, **2012**, *48*(11), 705-711. <http://dx.doi.org/10.1358/dot.2012.48.11.1871575> PMID: 23170306
- [174] Available from: <https://www.wepclinical.com/wep-clinical-partners-with-btg-specialty-pharmaceuticals-for-european-supply-of-voraxaze/>
- [175] Widemann, B.C.; Sung, E.; Anderson, L.; Salzer, W.L.; Balis, F.M.; Monitjo, K.S.; McCully, C.; Hawkins, M.; Adamson, P.C. Pharmacokinetics and metabolism of the methotrexate metabolite 2, 4-diamino-N(10)-methylptericoic acid. *J. Pharmacol. Exp. Ther.*, **2000**, *294*(3), 894-901. PMID: 10945838

- [176] Schwartz, S.; Borner, K.; Müller, K.; Martus, P.; Fischer, L.; Korfel, A.; Auton, T.; Thiel, E. Glucarpidase (carboxypeptidase g2) intervention in adult and elderly cancer patients with renal dysfunction and delayed methotrexate elimination after high-dose methotrexate therapy. *Oncologist*, **2007**, *12*(11), 1299-1308.
http://dx.doi.org/10.1634/theoncologist.12-11-1299 PMID: 18055849
- [177] Widemann, B.C.; Balis, F.M.; Kim, A.; Boron, M.; Jayaprakash, N.; Shalabi, A.; O'Brien, M.; Eby, M.; Cole, D.E.; Murphy, R.F.; Fox, E.; Ivy, P.; Adamson, P.C. Glucarpidase, leucovorin, and thymidine for high-dose methotrexate-induced renal dysfunction: Clinical and pharmacologic factors affecting outcome. *J. Clin. Oncol.*, **2010**, *28*(25), 3979-3986.
http://dx.doi.org/10.1200/JCO.2009.25.4540 PMID: 20679598
- [178] Widemann, B.C.; Balis, F.M.; Shalabi, A.; Boron, M.; O'Brien, M.; Cole, D.E.; Jayaprakash, N.; Ivy, P.; Castle, V.; Muraszko, K.; Moertel, C.L.; Trueworth, R.; Hermann, R.C.; Moussa, A.; Hinton, S.; Reaman, G.; Poplack, D.; Adamson, P.C. Treatment of accidental intrathecal methotrexate overdose with intrathecal carboxypeptidase G2. *J. Natl. Cancer Inst.*, **2004**, *96*(20), 1557-1559.
http://dx.doi.org/10.1093/jnci/djh270 PMID: 15494606
- [179] Available from: <https://www.who.int/health-topics/cardiovascular-diseases>
- [180] Undas, A.; Ariëns, R.A.S. Fibrin clot structure and function: A role in the pathophysiology of arterial and venous thromboembolic diseases. *Arterioscler. Thromb. Vasc. Biol.*, **2011**, *31*(12), e88-e99.
http://dx.doi.org/10.1161/ATVBAHA.111.230631 PMID: 21836064
- [181] Mosesson, M.W. Fibrinogen and fibrin structure and functions. *J. Thromb. Haemost.*, **2005**, *3*(8), 1894-1904.
http://dx.doi.org/10.1111/j.1538-7836.2005.01365.x PMID: 16102057
- [182] Chapin, J.C.; Hajjar, K.A. Fibrinolysis and the control of blood coagulation. *Blood Rev.*, **2015**, *29*(1), 17-24.
http://dx.doi.org/10.1016/j.blre.2014.09.003 PMID: 25294122
- [183] Mican, J.; Toul, M.; Bednar, D.; Damborsky, J. Structural Biology and Protein Engineering of Thrombolytics. *Comput. Struct. Biotechnol. J.*, **2019**, *17*, 917-938.
http://dx.doi.org/10.1016/j.csbj.2019.06.023 PMID: 31360331
- [184] Castellino, F.J.; Ploplis, V.A. Structure and function of the plasminogen/plasmin system. *Thromb. Haemost.*, **2005**, *93*(4), 647-654.
http://dx.doi.org/10.1160/TH04-12-0842 PMID: 15841308
- [185] Law, R.H.P.; Caradoc-Davies, T.; Cowieson, N.; Horvath, A.J.; Quek, A.J.; Encarnacao, J.A.; Steer, D.; Cowan, A.; Zhang, Q.; Lu, B.G.C.; Pike, R.N.; Smith, A.I.; Coughlin, P.B.; Whisstock, J.C. The X-ray crystal structure of full-length human plasminogen. *Cell Rep.*, **2012**, *1*(3), 185-190.
http://dx.doi.org/10.1016/j.celrep.2012.02.012 PMID: 22832192
- [186] Law, R.H.P.; Abu-Ssaydeh, D.; Whisstock, J.C. New insights into the structure and function of the plasminogen/plasmin system. *Curr. Opin. Struct. Biol.*, **2013**, *23*(6), 836-841.
http://dx.doi.org/10.1016/j.sbi.2013.10.006 PMID: 24252474
- [187] Irigoyen, J.P.; Muñoz-Cánoves, P.; Montero, L.; Koziczak, M.; Nagamine, Y. The plasminogen activator system: Biology and regulation. *Cell. Mol. Life Sci.*, **1999**, *56*(1-2), 104-132.
http://dx.doi.org/10.1007/PL00000615 PMID: 11213252
- [188] Urano, T.; Castellino, F.J.; Suzuki, Y. Regulation of plasminogen activation on cell surfaces and fibrin. *J. Thromb. Haemost.*, **2018**, *16*(8), 1487-1497.
http://dx.doi.org/10.1111/jth.14157 PMID: 29779246
- [189] Kotb, E. Activity assessment of microbial fibrinolytic enzymes. *Appl. Microbiol. Biotechnol.*, **2013**, *97*(15), 6647-6665.
http://dx.doi.org/10.1007/s00253-013-5052-1 PMID: 23812278
- [190] Kumar, S.S.; Sabu, A. *Therapeutic Enzymes: Function and Clinical Implications*; Labrou, N., Ed.; , **2019**, Vol. 1148, pp. 345-381.
http://dx.doi.org/10.1007/978-981-13-7709-9_15
- [191] Nihalani, D.; Sahni, G. Streptokinase contains two independent plasminogen-binding sites. *Biochem. Biophys. Res. Commun.*, **1995**, *217*(3), 1245-1254.
http://dx.doi.org/10.1006/bbrc.1995.2902 PMID: 8554583
- [192] Banerjee, A.; Chisti, Y.; Banerjee, U.C. Streptokinase--a clinically useful thrombolytic agent. *Biotechnol. Adv.*, **2004**, *22*(4), 287-307.
http://dx.doi.org/10.1016/j.biotechadv.2003.09.004 PMID: 14697452
- [193] Reed, G.L.; Hough, A.K.; Liu, L.; Parhami-Seren, B.; Matsueda, L.H.; Wang, S.; Hedstrom, L. A catalytic switch and the conversion of streptokinase to a fibrin-targeted plasminogen activator. *Proc. Natl. Acad. Sci. USA*, **1999**, *96*(16), 8879-8883.
http://dx.doi.org/10.1073/pnas.96.16.8879 PMID: 10430864
- [194] Sazonova, I.Y.; Robinson, B.R.; Gladysheva, I.P.; Castellino, F.J.; Reed, G.L. Alpha Domain deletion converts streptokinase into a fibrin-dependent plasminogen activator through mechanisms akin to staphylokinase and tissue plasminogen activator. *J. Biol. Chem.*, **2004**, *279*(24), 24994-25001.
http://dx.doi.org/10.1074/jbc.M400253200 PMID: 15069059
- [195] Kazmi, K.A.; Perwaiz Iqbal, M.; Rahbar, A.; Mehboobali, N. Anti-streptokinase titers and response to streptokinase treatment in Pakistani patients. *Int. J. Cardiol.*, **2002**, *82*(3), 247-251.
http://dx.doi.org/10.1016/S0167-5273(02)00004-9 PMID: 11911912
- [196] Sikri, N.; Bardia, A. A history of streptokinase use in acute myocardial infarction. *Tex. Heart Inst. J.*, **2007**, *34*(3), 318-327.
PMID: 17948083
- [197] (GISSI), G.I.p.I.S.d.S.n.I.M., Long-term effects of intravenous thrombolysis in acute myocardial infarction: Final report of the GISSI study. *Lancet*, **1987**, *2*, 871-874.
- [198] Available from: <https://www.medicines.org.uk/emc/product/4255/smpc>
- [199] Available from: <https://www.medicines.org.uk/emc/product/4256/smpc>
- [200] Available from: www.medicines.org.uk/emc/product/264/smpc
- [201] Available from: <https://www.medicines.org.uk/emc/product/898/smpc>
- [202] Available from: <https://www.medicines.org.uk/emc/product/3013/smpc>
- [203] Chester, K.W.; Corrigan, M.; Schoeffler, J.M.; Shah, M.; Toy, F.; Purdon, B.; Dillon, G.M. Making a case for the right '-ase' in acute ischemic stroke: Alteplase, tenecteplase, and reteplase. *Expert Opin. Drug Saf.*, **2019**, *18*(2), 87-96.

- <http://dx.doi.org/10.1080/14740338.2019.1573985> PMID: 30712409
- [204] Smith, R.A.G.; Dupe, R.J.; English, P.D.; Green, J. Fibrinolysis with acyl-enzymes: A new approach to thrombolytic therapy. *Nature*, **1981**, 290(5806), 505-508. <http://dx.doi.org/10.1038/290505a0> PMID: 7219537
- [205] Sherry, S. Pharmacology of anistreplase. *Clin. Cardiol.*, **1990**, 13(3)(Suppl. 5), V3-V10. <http://dx.doi.org/10.1002/clc.4960131303> PMID: 2182238
- [206] Adivitiya, ; Khasa, Y.P. The evolution of recombinant thrombolytics: Current status and future directions. *Bioengineered*, **2017**, 8(4), 331-358. <http://dx.doi.org/10.1080/21655979.2016.1229718> PMID: 27696935
- [207] MacFarlane, R.G.; Pilling, J. Fibrinolytic activity of normal urine. *Nature*, **1947**, 159(4049), 779. <http://dx.doi.org/10.1038/159779a0> PMID: 20241608
- [208] Crippa, M.P. Urokinase-type plasminogen activator. *Int. J. Biochem. Cell Biol.*, **2007**, 39(4), 690-694. <http://dx.doi.org/10.1016/j.biocel.2006.10.008> PMID: 17118695
- [209] Blasi, F.; Carmeliet, P. uPAR: A versatile signalling orchestrator. *Nat. Rev. Mol. Cell Biol.*, **2002**, 3(12), 932-943. <http://dx.doi.org/10.1038/nrm977> PMID: 12461559
- [210] Appella, E.; Blasi, F. The growth factor module of urokinase is the binding sequence for its receptor. *Ann. N. Y. Acad. Sci.*, **1987**, 511, 192-195. <http://dx.doi.org/10.1111/j.1749-6632.1987.tb36247.x> PMID: 2830824
- [211] Conese, M.; Blasi, F. Urokinase/urokinase receptor system: Internalization/degradation of urokinase-serpin complexes: Mechanism and regulation. *Biol. Chem. Hoppe Seyler*, **1995**, 376(3), 143-155. PMID: 7612191
- [212] Gurewich, V. Fibrinolysis: A Misunderstood Natural Defense Whose Therapeutic Potential Is Unknown. *Cardiovasc. Drugs Ther.*, **2019**, 33(6), 749-753. <http://dx.doi.org/10.1007/s10557-019-06923-8> PMID: 31897763
- [213] Kadir, R.R.A.; Bayraktutan, U. Urokinase Plasminogen Activator: A Potential Thrombolytic Agent for Ischaemic Stroke. *Cell. Mol. Neurobiol.*, **2020**, 40(3), 347-355. <http://dx.doi.org/10.1007/s10571-019-00737-w> PMID: 31552559
- [214] Astrup, T.; Permin, P.M. Fibrinolysis in the animal organism. *Nature*, **1947**, 159(4046), 681-682. <http://dx.doi.org/10.1038/159681b0> PMID: 20342264
- [215] Astrup, T.; Stage, A. Isolation of a soluble fibrinolytic activator from animal tissue. *Nature*, **1952**, 170(4335), 929. <http://dx.doi.org/10.1038/170929a0> PMID: 13013265
- [216] Collen, D.; Lijnen, H.R. The tissue-type plasminogen activator story. *Arterioscler. Thromb. Vasc. Biol.*, **2009**, 29(8), 1151-1155. <http://dx.doi.org/10.1161/ATVBAHA.108.179655> PMID: 19605778
- [217] Larsen, G.R.; Metzger, M.; Henson, K.; Blue, Y.; Horgan, P. Pharmacokinetic and distribution analysis of variant forms of tissue-type plasminogen activator with prolonged clearance in rat. *Blood*, **1989**, 73(7), 1842-1850. <http://dx.doi.org/10.1182/blood.V73.7.1842.1842> PMID: 2496774
- [218] Narita, M.; Bu, G.; Herz, J.; Schwartz, A.L. Two receptor systems are involved in the plasma clearance of tissue-type plasminogen activator (t-PA) *in vivo*. *J. Clin. Invest.*, **1995**, 96(2), 1164-1168. <http://dx.doi.org/10.1172/JCI118105> PMID: 7635954
- [219] Lijnen, H.R.; Collen, D. Strategies for the improvement of thrombolytic agents. *Thromb. Haemost.*, **1991**, 66(1), 88-110. <http://dx.doi.org/10.1055/s-0038-1646377> PMID: 1926054
- [220] Pennica, D.; Holmes, W.E.; Kohr, W.J.; Harkins, R.N.; Vihar, G.A.; Ward, C.A.; Bennett, W.F.; Yelverton, E.; Seeburg, P.H.; Heyneker, H.L.; Goeddel, D.V.; Collen, D. Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli. *Nature*, **1983**, 301(5897), 214-221. <http://dx.doi.org/10.1038/301214a0> PMID: 6337343
- [221] Hacke, W.; Kaste, M.; Bluhmki, E.; Brozman, M.; Dávalos, A.; Guidetti, D.; Larrue, V.; Lees, K.R.; Medeghri, Z.; Machnig, T.; Schneider, D.; von Kummer, R.; Wahlgren, N.; Toni, D.; Investigators, E. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N. Engl. J. Med.*, **2008**, 359(13), 1317-1329. <http://dx.doi.org/10.1056/NEJMoa0804656> PMID: 18815396
- [222] Leung, T.W.H.; Wong, K.S.L. Thrombolysis with alteplase for acute ischemic stroke: Safe and effective outside the 3-hour time window? *Nat. Clin. Pract. Neurol.*, **2009**, 5(2), 70-71. <http://dx.doi.org/10.1038/ncpneuro0993> PMID: 19107108
- [223] Choudhury, R.; Barrett, C.D.; Moore, H.B.; Moore, E.E.; McIntyre, R.C.; Moore, P.K.; Talmor, D.S.; Nydam, T.L.; Yaffe, M.B. Salvage use of tissue plasminogen activator (tPA) in the setting of acute respiratory distress syndrome (ARDS) due to COVID-19 in the USA: A Markov decision analysis. *World J. Emerg. Surg.*, **2020**, 15(1), 29. <http://dx.doi.org/10.1186/s13017-020-00305-4> PMID: 32312290
- [224] Dobesh, P.P.; Trujillo, T.C. Coagulopathy, Venous Thromboembolism, and Anticoagulation in Patients with COVID-19. *Pharmacotherapy*, **2020**, 40(11), 1130-1151. <http://dx.doi.org/10.1002/phar.2465> PMID: 33006163
- [225] Kollias, A.; Kyriakoulis, K.G.; Dimakakos, E.; Poulakou, G.; Stergiou, G.S.; Syrigos, K. Thromboembolic risk and anticoagulant therapy in COVID-19 patients: Emerging evidence and call for action. *Br. J. Haematol.*, **2020**, 189(5), 846-847. <http://dx.doi.org/10.1111/bjh.16727> PMID: 32304577
- [226] Paranjpe, I.; Fuster, V.; Lala, A.; Russak, A.J.; Glicksberg, B.S.; Levin, M.A.; Charney, A.W.; Narula, J.; Fayad, Z.A.; Bagiella, E.; Zhao, S.; Nadkarni, G.N. Association of Treatment Dose Anticoagulation With In-Hospital Survival Among Hospitalized Patients With COVID-19. *J. Am. Coll. Cardiol.*, **2020**, 76(1), 122-124. <http://dx.doi.org/10.1016/j.jacc.2020.05.001> PMID: 32387623
- [227] Horie, S.; McNicholas, B.; Rezoagli, E.; Pham, T.; Curley, G.; McAuley, D.; O'Kane, C.; Nichol, A.; Dos Santos, C.; Rocco, P.R.M.; Bellani, G.; Laffey, J.G. Emerging pharmacological therapies for ARDS: COVID-19 and beyond. *Intensive Care Med.*, **2020**, 46(12), 2265-2283. <http://dx.doi.org/10.1007/s00134-020-06141-z> PMID: 32654006
- [228] Ranucci, M.; Sitzia, C.; Baryshnikova, E.; Di Dedda, U.; Cardani, R.; Martelli, F.; Corsi Romanelli, M. Covid-19-Associated Coagulopathy: Biomarkers of Thrombin Generation and Fibrinolysis Leading the Outcome. *J. Clin. Med.*, **2020**, 9(11), E3487. <http://dx.doi.org/10.3390/jcm9113487> PMID: 33126772
- [229] Zhou, F.; Yu, T.; Du, R.; Fan, G.; Liu, Y.; Liu, Z.; Xiang, J.; Wang, Y.; Song, B.; Gu, X.; Guan, L.; Wei, Y.; Li, H.; Wu, X.; Xu, J.; Tu, S.; Zhang, Y.; Chen, H.; Cao, B. Clinical course and risk factors for mortality of adult inpatients with

- COVID-19 in Wuhan, China: A retrospective cohort study. *Lancet*, **2020**, 395(10229), 1054-1062.
[http://dx.doi.org/10.1016/S0140-6736\(20\)30566-3](http://dx.doi.org/10.1016/S0140-6736(20)30566-3) PMID: 32171076
- [230] Bikkeli, B.; Madhavan, M.V.; Gupta, A.; Jimenez, D.; Burton, J.R.; Der Nigoghossian, C.; Chuich, T.; Nouri, S.N.; Dreyfus, I.; Driggin, E.; Sethi, S.; Sehgal, K.; Chatterjee, S.; Ageno, W.; Madjid, M.; Guo, Y.; Tang, L.V.; Hu, Y.; Bertolotti, L.; Giri, J.; Cushman, M.; Quéré, I.; Dimakakos, E.P.; Gibson, C.M.; Lippi, G.; Favaloro, E.J.; Fareed, J.; Taffur, A.J.; Francese, D.P.; Batra, J.; Falanga, A.; Clerkin, K.J.; Uriel, N.; Kirtane, A.; McLintock, C.; Hunt, B.J.; Spyropoulos, A.C.; Barnes, G.D.; Eikelboom, J.W.; Weinberg, I.; Schulman, S.; Carrier, M.; Piazza, G.; Beckman, J.A.; Leon, M.B.; Stone, G.W.; Rosenkranz, S.; Goldhaber, S.Z.; Parikh, S.A.; Monreal, M.; Krumholz, H.M.; Konstantinides, S.V.; Weitz, J.I.; Lip, G.Y.H.; Global, C-T.C. Pharmacological Agents Targeting Thromboinflammation in COVID-19: Review and Implications for Future Research. *Thromb. Haemost.*, **2020**, 120(7), 1004-1024.
<http://dx.doi.org/10.1055/s-0040-1713152> PMID: 32473596
- [231] Moore, H.B.; Barrett, C.D.; Moore, E.E.; McIntyre, R.C.; Moore, P.K.; Talmor, D.S.; Moore, F.A.; Yaffe, M.B. Is there a role for tissue plasminogen activator as a novel treatment for refractory COVID-19 associated acute respiratory distress syndrome? *J. Trauma Acute Care Surg.*, **2020**, 88(6), 713-714.
<http://dx.doi.org/10.1097/TA.0000000000002694> PMID: 32281766
- [232] Hardaway, R.M.; Harke, H.; Tyroch, A.H.; Williams, C.H.; Vazquez, Y.; Krause, G.F. Treatment of severe acute respiratory distress syndrome: A final report on a phase I study. *Am. Surg.*, **2001**, 67(4), 377-382.
 PMID: 11308009
- [233] Abdelaal Ahmed Mahmoud, A.; Mahmoud, H.E.; Mahran, M.A.; Khaled, M. Streptokinase Versus Unfractionated Heparin Nebulization in Patients With Severe Acute Respiratory Distress Syndrome (ARDS): A Randomized Controlled Trial With Observational Controls. *J. Cardiothorac. Vasc. Anesth.*, **2020**, 34(2), 436-443.
<http://dx.doi.org/10.1053/j.jvca.2019.05.035> PMID: 31262641
- [234] Goyal, A.; Saigal, S.; Niwariya, Y.; Sharma, J.; Singh, P. Successful use of tPA for thrombolysis in COVID related ARDS: A case series. *J. Thromb. Thrombolysis*, **2020**.
<http://dx.doi.org/10.1007/s11239-020-02208-2> PMID: 32617806
- [235] Poor, H.D.; Ventetuolo, C.E.; Tolbert, T.; Chun, G.; Serrao, G.; Zeidman, A.; Dangayach, N.S.; Olin, J.; Kohli-Seth, R.; Powell, C.A. COVID-19 critical illness pathophysiology driven by diffuse pulmonary thrombi and pulmonary endothelial dysfunction responsive to thrombolysis. *Clin. Transl. Med.*, **2020**, 10(2), e44.
<http://dx.doi.org/10.1002/ctm2.44> PMID: 32508062
- [236] Wang, J.; Hajizadeh, N.; Moore, E.E.; McIntyre, R.C.; Moore, P.K.; Veress, L.A.; Yaffe, M.B.; Moore, H.B.; Barrett, C.D. Tissue plasminogen activator (tPA) treatment for COVID-19 associated acute respiratory distress syndrome (ARDS): A case series. *J. Thromb. Haemost.*, **2020**, 18(7), 1752-1755.
<http://dx.doi.org/10.1111/jth.14828> PMID: 32267998
- [237] Carneiro, T.; Dashkoff, J.; Leung, L.Y.; Nobleza, C.O.S.; Marulanda-Londono, E.; Hathidara, M.; Koch, S.; Sur, N.; Boske, A.; Voetsch, B.; Aboul Nour, H.; Miller, D.J.; Daneshmand, A.; Shulman, J.; Curiale, G.; Greer, D.M.; Romero, J.R.; Anand, P.; Cervantes-Arslanian, A.M. Intravenous tPA for Acute Ischemic Stroke in Patients with COVID-19. *J. Stroke Cerebrovasc. Dis.*, **2020**, 29(11), 105201.
<http://dx.doi.org/10.1016/j.jstrokecerebrovasdis.2020.105201> PMID: 33066885
- [238] Keyt, B.A.; Paoni, N.F.; Refino, C.J.; Berleau, L.; Nguyen, H.; Chow, A.; Lai, J.; Peña, L.; Pater, C.; Ogez, J.; Etcheverry, T.; Botstein, D.; Bennett, W.F. A faster-acting and more potent form of tissue plasminogen activator. *Proc. Natl. Acad. Sci. USA*, **1994**, 91(9), 3670-3674.
<http://dx.doi.org/10.1073/pnas.91.9.3670> PMID: 8170967
- [239] Cannon, C.P.; McCabe, C.H.; Gibson, C.M.; Ghali, M.; Sequeira, R.F.; McKendall, G.R.; Breed, J.; Modi, N.B.; Fox, N.L.; Tracy, R.P.; Love, T.W.; Braunwald, E.; Coulson, T.; Mayor, M.R.; Girwarr, G.; Teixeira, P.; Williams, D.O.; McDonald, M.; Kirshenbaum, J.M.; Cloutier, J.; Henry, T.D.; Knox, L.; Boisjolie, C.; Schweiger, M.J.; Burkott, B.; Warwick, D.; Carney, R.; Murphy, G.; Cutler, D.; Malmberg, G.; Greene, R.M.; Healy, E.; Perry, V.; Watkins, M.; Sobel, B.E.; Rowen, M.; Wilson, W.; Cuttitta, J.; Niederman, A.; Kellerman, T.; Frey, M.; Taylor, H.; Mueller, H.S.; Kunkel, L.; Cosico, J.; Casale, P.; Tuzi, J.; Rogers, W.J.; Morgan, T.; Anderson, J.L.; Karagounis, L.; Allen, A.; Hochman, J.S.; McAnulty, M. TNK-tissue plasminogen activator in acute myocardial infarction. Results of the Thrombolysis in Myocardial Infarction (TIMI) 10A dose-ranging trial. *Circulation*, **1997**, 95(2), 351-356.
<http://dx.doi.org/10.1161/01.CIR.95.2.351> PMID: 9008448
- [240] Zeymer, U.; Neuhaus, K.L. Clinical trials in acute myocardial infarction. *Curr. Opin. Cardiol.*, **1999**, 14(5), 392-402.
<http://dx.doi.org/10.1097/00001573-199909000-00007> PMID: 10500901
- [241] Kohnert, U.; Rudolph, R.; Verheijen, J.H.; Weening-Verhoeff, E.J.D.; Stern, A.; Opitz, U.; Martin, U.; Lill, H.; Prinz, H.; Lechner, M.; Kresse, G.B.; Buckel, P.; Fischer, S. Biochemical properties of the kringle 2 and protease domains are maintained in the refolded t-PA deletion variant BM 06.022. *Protein Eng.*, **1992**, 5(1), 93-100.
<http://dx.doi.org/10.1093/protein/5.1.93> PMID: 1321420
- [242] Malcolm, A.D.; Keltai, M.; Walsh, M.J.; Hennesdorf, G.; Dymond, D.; Fabian, J.; Sochman, J.; Bertrand, M.; Masquet, C.; Letac, B.; Bory, M.; Eha, J.; DeVita, C.; Ravazzi, P.; Gatto, E.; Abbadessa, F.; Perrins, J.; Walsh, M.; Daly, K. ESPRIT: A European study of the prevention of reocclusion after initial thrombolysis with alteplase in acute myocardial infarction. *Eur. Heart J.*, **1996**, 17(10), 1522-1531.
<http://dx.doi.org/10.1093/oxfordjournals.eurheartj.a014716> PMID: 8909909
- [243] Martin, U.; von Möllendorff, E.; Akpan, W.; Kientsch-Engel, R.; Kaufmann, B.; Neugebauer, G. Pharmacokinetic and hemostatic properties of the recombinant plasminogen activator bm 06.022 in healthy volunteers. *Thromb. Haemost.*, **1991**, 66(5), 569-574.
<http://dx.doi.org/10.1055/s-0038-1646461> PMID: 1725068
- [244] Oikawa, K.; Watanabe, T.; Higuchi, S. Comparison of drug disposition between wild-type and novel tissue-type plasminogen activator pamiteplase in rats. *Drug Metab. Dispos.*, **2000**, 28(9), 1087-1093.
 PMID: 10950854
- [245] Oikawa, K.; Watanabe, T.; Miyamoto, I.; Higuchi, S. Determination, pharmacokinetics and protein binding of a novel tissue-type plasminogen activator, pamiteplase in human plasma. *Xenobiotica*, **2000**, 30(10), 993-1003.
<http://dx.doi.org/10.1080/00498250050200140> PMID: 11315107

- [246] Agnelli, G.; Pascucci, C.; Nenci, G.G.; Mele, A.; Bürgi, R.; Heim, J. Thrombolytic and haemorrhagic effects of bolus doses of tissue-type plasminogen activator and a hybrid plasminogen activator with prolonged plasma half-life (K2tu-PA: CGP 42935). *Thromb. Haemost.*, **1993**, 70(2), 294-300.
<http://dx.doi.org/10.1055/s-0038-1649569> PMID: 8236138
- [247] Nedaeinia, R.; Faraji, H.; Javanmard, S.H.; Ferns, G.A.; Ghayour-Mobarhan, M.; Goli, M.; Mashkani, B.; Nedaeinia, M.; Haghighi, M.H.H.; Ranjbar, M. Bacterial staphylokinase as a promising third-generation drug in the treatment for vascular occlusion. *Mol. Biol. Rep.*, **2020**, 47(1), 819-841.
<http://dx.doi.org/10.1007/s11033-019-05167-x> PMID: 31677034
- [248] Hawkey, C. Plasminogen activator in saliva of the vampire bat *Desmodus rotundus*. *Nature*, **1966**, 211(5047), 434-435.
<http://dx.doi.org/10.1038/211434c0> PMID: 5967844
- [249] Krätzschmar, J.; Haendler, B.; Langer, G.; Boidol, W.; Bringmann, P.; Alagon, A.; Donner, P.; Schleuning, W.D. The plasminogen activator family from the salivary gland of the vampire bat *Desmodus rotundus*: Cloning and expression. *Gene*, **1991**, 105(2), 229-237.
[http://dx.doi.org/10.1016/0378-1119\(91\)90155-5](http://dx.doi.org/10.1016/0378-1119(91)90155-5) PMID: 1937019
- [250] Schleuning, W.D.; Alagon, A.; Boidol, W.; Bringmann, P.; Petri, T.; Krätzschmar, J.; Haendler, B.; Langer, G.; Baldus, B.; Witt, W.; Donner, P. Plasminogen activators from the saliva of *Desmodus rotundus* (common vampire bat): Unique fibrin specificity. *Ann. N. Y. Acad. Sci.*, **1992**, 667, 395-403.
<http://dx.doi.org/10.1111/j.1749-6632.1992.tb51639.x> PMID: 1309059
- [251] Bringmann, P.; Gruber, D.; Liese, A.; Toschi, L.; Krätzschmar, J.; Schleuning, W.D.; Donner, P. Structural features mediating fibrin selectivity of vampire bat plasminogen activators. *J. Biol. Chem.*, **1995**, 270(43), 25596-25603.
<http://dx.doi.org/10.1074/jbc.270.43.25596> PMID: 7592732
- [252] Medcalf, R.L. Desmoteplase: Discovery, insights and opportunities for ischaemic stroke. *Br. J. Pharmacol.*, **2012**, 165(1), 75-89.
<http://dx.doi.org/10.1111/j.1476-5381.2011.01514.x> PMID: 21627637
- [253] Schleuning, W.D. Vampire bat plasminogen activator DSPA- α -1 (desmoteplase): A thrombolytic drug optimized by natural selection. *Haemostasis*, **2001**, 31(3-6), 118-122.
PMID: 11910176
- [254] Hacke, W.; Furlan, A.J.; Al-Rawi, Y.; Davalos, A.; Fiebach, J.B.; Gruber, F.; Kaste, M.; Lipka, L.J.; Pedraza, S.; Ringleb, P.A.; Rowley, H.A.; Schneider, D.; Schwamm, L.H.; Leal, J.S.; Sönnngen, M.; Teal, P.A.; Wilhelm-Ogunbiyi, K.; Wintermark, M.; Warach, S. Intravenous desmoteplase in patients with acute ischaemic stroke selected by MRI perfusion-diffusion weighted imaging or perfusion CT (DIAS-2): A prospective, randomised, double-blind, placebo-controlled study. *Lancet Neurol.*, **2009**, 8(2), 141-150.
[http://dx.doi.org/10.1016/S1474-4422\(08\)70267-9](http://dx.doi.org/10.1016/S1474-4422(08)70267-9) PMID: 19097942
- [255] von Kummer, R.; Mori, E.; Truelsen, T.; Jensen, J.S.; Grønning, B.A.; Fiebach, J.B.; Lovblad, K.O.; Pedraza, S.; Romero, J.M.; Chabriat, H.; Chang, K.C.; Dávalos, A.; Ford, G.A.; Grotta, J.; Kaste, M.; Schwamm, L.H.; Shuaib, A.; Albers, G.W.; Investigators, D. Desmoteplase 3 to 9 Hours After Major Artery Occlusion Stroke: The DIAS-4 Trial (Efficacy and Safety Study of Desmoteplase to Treat Acute Ischemic Stroke). *Stroke*, **2016**, 47(12), 2880-2887.
<http://dx.doi.org/10.1161/STROKEAHA.116.013715> PMID: 27803391
- [256] Rabijs, A.; De Bondt, H.L.; De Ranter, C. Three-dimensional structure of staphylokinase, a plasminogen activator with therapeutic potential. *Nat. Struct. Biol.*, **1997**, 4(5), 357-360.
<http://dx.doi.org/10.1038/nsb0597-357> PMID: 9145104
- [257] Marder, V.J.; Novokhatny, V. Direct fibrinolytic agents: Biochemical attributes, preclinical foundation and clinical potential. *J. Thromb. Haemost.*, **2010**, 8(3), 433-444.
<http://dx.doi.org/10.1111/j.1538-7836.2009.03701.x> PMID: 19943877
- [258] Marder, V.J. Historical perspective and future direction of thrombolysis research: The re-discovery of plasmin. *J. Thromb. Haemost.*, **2011**, 9(Suppl. 1), 364-373.
<http://dx.doi.org/10.1111/j.1538-7836.2011.04370.x> PMID: 21781273
- [259] Hoefler, I.E.; Stroes, E.S.G.; Pasterkamp, G.; Levi, M.M.; Reekers, J.A.; Verhagen, H.J.M.; Meijers, J.C.; Humphries, J.E.; Rotmans, J.I. Locally applied recombinant plasmin results in effective thrombolysis in a porcine model of arteriovenous graft thrombosis. *J. Vasc. Interv. Radiol.*, **2009**, 20(7), 951-958.
<http://dx.doi.org/10.1016/j.jvir.2009.03.043> PMID: 19481472
- [260] Shlansky-Goldberg, R.D.; Matsumoto, A.H.; Baumbach, G.A.; Siegel, J.B.; Raabe, R.D.; Murphy, T.P.; Deng, C.; Ray Dawkins, J.; Marder, V.J. A first-in-human phase I trial of locally delivered human plasmin for hemodialysis graft occlusion. *J. Thromb. Haemost.*, **2008**, 6(6), 944-950.
<http://dx.doi.org/10.1111/j.1538-7836.2008.02969.x> PMID: 18384651
- [261] Fu, L.H.; Qi, C.; Lin, J.; Huang, P. Catalytic chemistry of glucose oxidase in cancer diagnosis and treatment. *Chem. Soc. Rev.*, **2018**, 47(17), 6454-6472.
<http://dx.doi.org/10.1039/C7CS00891K> PMID: 30024579
- [262] Lin, X.; Wang, Y.; Zhang, Y.; Huang, B.; Lin, J.J.; Hallock, S.J.; Yu, H.; Shao, H.; Yan, J.; Huang, B.; Zhang, X.C.J.; Cao, W.; Xu, X.; Lin, X. Purification and characterization of mutant miniPlasmin for thrombolytic therapy. *Thromb. J.*, **2013**, 11(1), 2.
<http://dx.doi.org/10.1186/1477-9560-11-2> PMID: 23363549
- [263] Nagai, N.; Demarsin, E.; Van Hoef, B.; Wouters, S.; Cingolani, D.; Laroche, Y.; Collen, D. Recombinant human microplasmin: Production and potential therapeutic properties. *J. Thromb. Haemost.*, **2003**, 1(2), 307-313.
<http://dx.doi.org/10.1046/j.1538-7836.2003.00078.x> PMID: 12871505
- [264] Thijs, V.N.S.; Peeters, A.; Vosko, M.; Aichner, F.; Schellinger, P.D.; Schneider, D.; Neumann-Haefelin, T.; Röther, J.; Davalos, A.; Wahlgren, N.; Verhamme, P. Randomized, placebo-controlled, dose-ranging clinical trial of intravenous microplasmin in patients with acute ischemic stroke. *Stroke*, **2009**, 40(12), 3789-3795.
<http://dx.doi.org/10.1161/STROKEAHA.109.560201> PMID: 19834019
- [265] Kaur, N.; Sinha, P.K.; Sahni, G. Site-specific PEGylation of micro-plasmin for improved thrombolytic therapy through engineering enhanced resistance against serpin mediated inhibition. *PLoS One*, **2019**, 14(5), e0217234.
<http://dx.doi.org/10.1371/journal.pone.0217234> PMID: 31141522
- [266] Chen, W.; Huang, X.; Ma, X.W.; Mo, W.; Wang, W.J.; Song, H.Y. Enzymatic vitreolysis with recombinant microplasminogen and tissue plasminogen activator. *Eye (Lond.)*, **2008**, 22(2), 300-307.

- <http://dx.doi.org/10.1038/sj.eye.6702931> PMID: 17704761
- [267] de Smet, M.D.; Valmaggia, C.; Zarranz-Ventura, J.; Willekens, B. Microplasmin: Ex vivo characterization of its activity in porcine vitreous. *Invest. Ophthalmol. Vis. Sci.*, **2009**, *50*(2), 814-819.
<http://dx.doi.org/10.1167/iov.08-2185> PMID: 18806295
- [268] Haller, J.A.; Stalmans, P.; Benz, M.S.; Gandorfer, A.; Pakola, S.J.; Girach, A.; Kampik, A.; Jaffe, G.J.; Toth, C.A.; Grp, M-T.S. Efficacy of intravitreal ocriplasmin for treatment of vitreomacular adhesion: Subgroup analyses from two randomized trials. *Ophthalmology*, **2015**, *122*(1), 117-122.
<http://dx.doi.org/10.1016/j.opht.2014.07.045> PMID: 25240630
- [269] de Smet, M.D.; Stassen, J.M.; Meenink, T.C.M.; Janssens, T.; Vanheukelom, V.; Naus, G.J.L.; Beelen, M.J.; Jonckx, B. Release of experimental retinal vein occlusions by direct intraluminal injection of ocriplasmin. *Br. J. Ophthalmol.*, **2016**, *100*(12), 1742-1746.
<http://dx.doi.org/10.1136/bjophthalmol-2016-309190> PMID: 27688592
- [270] Willekens, K.; Gijbels, A.; Smits, J.; Schoevaerdt, L.; Blanckaert, J.; Feyen, J.H.M.; Reynaerts, D.; Stalmans, P. Phase I trial on robot assisted retinal vein cannulation with ocriplasmin infusion for central retinal vein occlusion. *Acta Ophthalmologica*,
- [271] Hunt, J.A.; Petteway, S.R., Jr; Scuderi, P.; Novokhatny, V. Simplified recombinant plasmin: Production and functional comparison of a novel thrombolytic molecule with plasma-derived plasmin. *Thromb. Haemost.*, **2008**, *100*(3), 413-419.
<http://dx.doi.org/10.1160/TH08-04-0225> PMID: 18766256
- [272] Sumi, H.; Hamada, H.; Tsushima, H.; Mihara, H.; Muraki, H. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet. *Experientia*, **1987**, *43*(10), 1110-1111.
<http://dx.doi.org/10.1007/BF01956052> PMID: 3478223
- [273] Yatagai, C.; Maruyama, M.; Kawahara, T.; Sumi, H. Nattokinase-promoted tissue plasminogen activator release from human cells. *Pathophysiol. Haemost. Thromb.*, **2008**, *36*(5), 227-232.
<http://dx.doi.org/10.1159/000252817> PMID: 19996631
- [274] Urano, T.; Ihara, H.; Umemura, K.; Suzuki, Y.; Oike, M.; Akita, S.; Tsukamoto, Y.; Suzuki, I.; Takada, A. The profibrinolytic enzyme subtilisin NAT purified from *Bacillus subtilis* Cleaves and inactivates plasminogen activator inhibitor type 1. *J. Biol. Chem.*, **2001**, *276*(27), 24690-24696.
<http://dx.doi.org/10.1074/jbc.M101751200> PMID: 11325965
- [275] Fujita, M.; Ito, Y.; Hong, K.; Nishimuro, S. Characterization of nattokinase-degraded products from human fibrinogen or cross-linked fibrin. *Fibrinolysis*, **1995**, *9*(3), 157-164.
[http://dx.doi.org/10.1016/S0268-9499\(95\)80005-0](http://dx.doi.org/10.1016/S0268-9499(95)80005-0)
- [276] Sumi, H.; Hamada, H.; Nakanishi, K.; Hiratani, H. Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. *Acta Haematol.*, **1990**, *84*(3), 139-143.
<http://dx.doi.org/10.1159/000205051> PMID: 2123064
- [277] Fujita, M.; Nomura, K.; Hong, K.; Ito, Y.; Asada, A.; Nishimuro, S. Purification and characterization of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan. *Biochem. Biophys. Res. Commun.*, **1993**, *197*(3), 1340-1347.
<http://dx.doi.org/10.1006/bbrc.1993.2624> PMID: 8280151
- [278] Hsia, C.H.; Shen, M.C.; Lin, J.S.; Wen, Y.K.; Hwang, K.L.; Cham, T.M.; Yang, N.C. Nattokinase decreases plasma levels of fibrinogen, factor VII, and factor VIII in human subjects. *Nutr. Res.*, **2009**, *29*(3), 190-196.
<http://dx.doi.org/10.1016/j.nutres.2009.01.009> PMID: 19358933
- [279] Kurosawa, Y.; Nirengi, S.; Homma, T.; Esaki, K.; Ohta, M.; Clark, J.F.; Hamaoka, T. A single-dose of oral nattokinase potentiates thrombolysis and anti-coagulation profiles. *Sci. Rep.*, **2015**, *5*, 11601.
<http://dx.doi.org/10.1038/srep11601> PMID: 26109079
- [280] Jensen, G.S.; Lenninger, M.; Ero, M.P.; Benson, K.F. Consumption of nattokinase is associated with reduced blood pressure and von Willebrand factor, a cardiovascular risk marker: Results from a randomized, double-blind, placebo-controlled, multicenter North American clinical trial. *Integr. Blood Press. Control*, **2016**, *9*, 95-104.
<http://dx.doi.org/10.2147/IBPC.S99553> PMID: 27785095
- [281] Pham, P.T.; Han, B.; Hoang, B.X. Nattospes as Effective and Safe Functional Supplements in Management of Stroke. *J. Med. Food*, **2020**, *23*(8), 879-885.
<http://dx.doi.org/10.1089/jmf.2019.0183> PMID: 31934821
- [282] Chen, H.; McGowan, E.M.; Ren, N.; Lal, S.; Nassif, N.; Shad-Kaneez, F.; Qu, X.; Lin, Y. Nattokinase: A Promising Alternative in Prevention and Treatment of Cardiovascular Diseases. *Biomark. Insights*, **2018**, *13*, 1177271918785130.
<http://dx.doi.org/10.1177/1177271918785130> PMID: 30013308
- [283] Lichter-Konecki, U.; Vockley, J. Phenylketonuria: Current Treatments and Future Developments. *Drugs*, **2019**, *79*(5), 495-500.
<http://dx.doi.org/10.1007/s40265-019-01079-z> PMID: 30864096
- [284] Al Hafid, N.; Christodoulou, J. Phenylketonuria: A review of current and future treatments. *Transl. Pediatr.*, **2015**, *4*(4), 304-317.
PMID: 26835392
- [285] Vockley, J.; Andersson, H.C.; Antshel, K.M.; Braverman, N.E.; Burton, B.K.; Frazier, D.M.; Mitchell, J.; Smith, W.E.; Thompson, B.H.; Berry, S.A. Phenylalanine hydroxylase deficiency: Diagnosis and management guideline. *Genet. Med.*, **2014**, *16*(2), 188-200.
<http://dx.doi.org/10.1038/gim.2013.157> PMID: 24385074
- [286] Mitchell, J.J.; Trakadis, Y.J.; Scriver, C.R. Phenylalanine hydroxylase deficiency. *Genet. Med.*, **2011**, *13*(8), 697-707.
<http://dx.doi.org/10.1097/GIM.0b013e3182141b48> PMID: 21555948
- [287] Hydery, T.; Coppenrath, V.A. A Comprehensive Review of Pegvaliase, an Enzyme Substitution Therapy for the Treatment of Phenylketonuria. *Drug Target Insights*, **2019**, *13*, 1177392819857089.
<http://dx.doi.org/10.1177/1177392819857089> PMID: 31258325
- [288] Hausmann, O.; Dahan, M.; Longo, N.; Knol, E.; Müller, I.; Northrup, H.; Brockow, K. Pegvaliase: Immunological profile and recommendations for the clinical management of hypersensitivity reactions in patients with phenylketonuria treated with this enzyme substitution therapy. *Mol. Genet. Metab.*, **2019**, *128*(1-2), 84-91.
<http://dx.doi.org/10.1016/j.ymgme.2019.05.006> PMID: 31375398
- [289] Ronda, L.; Marchetti, M.; Piano, R.; Liuzzi, A.; Corsini, R.; Percudani, R.; Bettati, S. A Trivalent Enzymatic System for Uricolytic Therapy of HPRT Deficiency and Lesch-Nyhan Disease. *Pharm. Res.*, **2017**, *34*(7), 1477-1490.
<http://dx.doi.org/10.1007/s11095-017-2167-6> PMID: 28508122

- [290] Pfister, D.; Morbidelli, M. Process for protein PEGylation. *J. Control. Release*, **2014**, *180*, 134-149.
<http://dx.doi.org/10.1016/j.jconrel.2014.02.002> PMID: 24531008
- [291] Pasut, G.; Veronese, F.M. State of the art in PEGylation: The great versatility achieved after forty years of research. *J. Control. Release*, **2012**, *161*(2), 461-472.
<http://dx.doi.org/10.1016/j.jconrel.2011.10.037> PMID: 22094104
- [292] Kontos, S.; Hubbell, J.A. Drug development: Longer-lived proteins. *Chem. Soc. Rev.*, **2012**, *41*(7), 2686-2695.
<http://dx.doi.org/10.1039/c2cs15289d> PMID: 22310725
- [293] Hubbell, J.A. Drug development: Longer-lived proteins. *Nature*, **2010**, *467*(7319), 1051-1052.
<http://dx.doi.org/10.1038/4671051a> PMID: 20981086
- [294] Sarkissian, C.N.; Shao, Z.; Blain, F.; Peevers, R.; Su, H.; Heft, R.; Chang, T.M.; Scriver, C.R. A different approach to treatment of phenylketonuria: Phenylalanine degradation with recombinant phenylalanine ammonia lyase. *Proc. Natl. Acad. Sci. USA*, **1999**, *96*(5), 2339-2344.
<http://dx.doi.org/10.1073/pnas.96.5.2339> PMID: 10051643
- [295] Moffitt, M.C.; Louie, G.V.; Bowman, M.E.; Pence, J.; Noel, J.P.; Moore, B.S. Discovery of two cyanobacterial phenylalanine ammonia lyases: Kinetic and structural characterization. *Biochemistry*, **2007**, *46*(4), 1004-1012.
<http://dx.doi.org/10.1021/bi061774g> PMID: 17240984
- [296] Mays, Z.J.; Mohan, K.; Trivedi, V.D.; Chappell, T.C.; Nair, N.U. Directed evolution of *Anabaena variabilis* phenylalanine ammonia-lyase (PAL) identifies mutants with enhanced activities. *Chem. Commun. (Camb.)*, **2020**, *56*(39), 5255-5258.
<http://dx.doi.org/10.1039/D0CC00783H> PMID: 32270162
- [297] Isabella, V.M.; Ha, B.N.; Castillo, M.J.; Lubkowitz, D.J.; Rowe, S.E.; Millet, Y.A.; Anderson, C.L.; Li, N.; Fisher, A.B.; West, K.A.; Reeder, P.J.; Momin, M.M.; Bergeron, C.G.; Guilmain, S.E.; Miller, P.F.; Kurtz, C.B.; Falb, D. Development of a synthetic live bacterial therapeutic for the human metabolic disease phenylketonuria. *Nat. Biotechnol.*, **2018**, *36*(9), 857-864.
<http://dx.doi.org/10.1038/nbt.4222> PMID: 30102294
- [298] McIntosh, I.; Cutting, G.R. Cystic fibrosis transmembrane conductance regulator and the etiology and pathogenesis of cystic fibrosis. *FASEB J.*, **1992**, *6*(10), 2775-2782.
<http://dx.doi.org/10.1096/fasebj.6.10.1378801> PMID: 1378801
- [299] Rowe, S.M.; Miller, S.; Sorscher, E.J. Cystic fibrosis. *N. Engl. J. Med.*, **2005**, *352*(19), 1992-2001.
<http://dx.doi.org/10.1056/NEJMra043184> PMID: 15888700
- [300] O'Sullivan, B.P.; Freedman, S.D. Cystic fibrosis. *Lancet*, **2009**, *373*(9678), 1891-1904.
- [301] Elborn, J.S. Cystic fibrosis. *Lancet*, **2016**, *388*(10059), 2519-2531.
PMID: 27140670
- [302] Ong, T.; Ramsey, B.W. Update in Cystic Fibrosis 2014. *Am. J. Respir. Crit. Care Med.*, **2015**, *192*(6), 669-675.
<http://dx.doi.org/10.1164/rccm.201504-0656UP> PMID: 26371812
- [303] Nazareth, D.; Walshaw, M. Coming of age in cystic fibrosis - transition from paediatric to adult care. *Clin. Med. (Lond.)*, **2013**, *13*(5), 482-486.
<http://dx.doi.org/10.7861/clinmedicine.13-5-482> PMID: 24115706
- [304] Sheppard, D.N.; Welsh, M.J. Structure and function of the CFTR chloride channel. *Physiol. Rev.*, **1999**, *79*(1)(Suppl.), S23-S45.
<http://dx.doi.org/10.1152/physrev.1999.79.1.S23> PMID: 9922375
- [305] Bobadilla, J.L.; Macek, M., Jr; Fine, J.P.; Farrell, P.M. Cystic fibrosis: A worldwide analysis of CFTR mutations--correlation with incidence data and application to screening. *Hum. Mutat.*, **2002**, *19*(6), 575-606.
<http://dx.doi.org/10.1002/humu.10041> PMID: 12007216
- [306] Sharma, J.; Keeling, K.M.; Rowe, S.M. Pharmacological approaches for targeting cystic fibrosis nonsense mutations. *Eur. J. Med. Chem.*, **2020**, *200*, 112436.
<http://dx.doi.org/10.1016/j.ejmech.2020.112436> PMID: 32512483
- [307] Guimbellot, J.; Sharma, J.; Rowe, S.M. Toward inclusive therapy with CFTR modulators: Progress and challenges. *Pediatr. Pulmonol.*, **2017**, *52*(S48), S4-S14.
<http://dx.doi.org/10.1002/ppul.23773> PMID: 28881097
- [308] Elborn, J.S.; Davies, J. Clinical trial research in focus: Ensuring new cystic fibrosis drugs fulfil their potential. *Lancet Respir. Med.*, **2017**, *5*(9), 681-683.
[http://dx.doi.org/10.1016/S2213-2600\(17\)30311-9](http://dx.doi.org/10.1016/S2213-2600(17)30311-9) PMID: 28853396
- [309] Birket, S.E.; Chu, K.K.; Houser, G.H.; Liu, L.; Fernandez, C.M.; Solomon, G.M.; Lin, V.; Shastry, S.; Mazur, M.; Sloane, P.A.; Hanes, J.; Grizzle, W.E.; Sorscher, E.J.; Tearney, G.J.; Rowe, S.M. Combination therapy with cystic fibrosis transmembrane conductance regulator modulators augment the airway functional microanatomy. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **2016**, *310*(10), L928-L939.
<http://dx.doi.org/10.1152/ajplung.00395.2015> PMID: 26968770
- [310] Yang, C.; Montgomery, M. Dornase alfa for cystic fibrosis. *Cochrane Database Syst. Rev.*, **2018**, *9*, CD001127.
PMID: 30187450
- [311] Yang, C.L.; Chilvers, M.; Montgomery, M.; Nolan, S.J. Dornase alfa for cystic fibrosis. *Paediatr. Respir. Rev.*, **2017**, *21*, 65-67.
PMID: 27769785
- [312] Lieberman, J. Dornase aerosol effect on sputum viscosity in cases of cystic fibrosis. *JAMA*, **1968**, *205*(5), 312-313.
<http://dx.doi.org/10.1001/jama.1968.03140310070022> PMID: 5694947
- [313] Cramer, G.W.; Bosso, J.A. The role of dornase alfa in the treatment of cystic fibrosis. *Ann. Pharmacother.*, **1996**, *30*(6), 656-661.
<http://dx.doi.org/10.1177/106002809603000614> PMID: 8792953
- [314] Parsiegla, G.; Noguere, C.; Santell, L.; Lazarus, R.A.; Bourne, Y. The structure of human DNase I bound to magnesium and phosphate ions points to a catalytic mechanism common to members of the DNase I-like superfamily. *Biochemistry*, **2012**, *51*(51), 10250-10258.
<http://dx.doi.org/10.1021/bi300873f> PMID: 23215638
- [315] Collier, J. Dornase-alfa and orphan drugs. *Lancet*, **1995**, *346*(8975), 633-633.
[http://dx.doi.org/10.1016/S0140-6736\(95\)91460-9](http://dx.doi.org/10.1016/S0140-6736(95)91460-9) PMID: 7651014
- [316] Shak, S.; Capon, D.J.; Hellmiss, R.; Marsters, S.A.; Baker, C.L. Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. *Proc. Natl. Acad. Sci. USA*, **1990**, *87*(23), 9188-9192.
<http://dx.doi.org/10.1073/pnas.87.23.9188> PMID: 2251263
- [317] Erdev, O.; Uras, N.; Atasay, B.; Arsan, S. Efficacy and safety of nebulized recombinant human DNase as rescue treatment for persistent atelectasis in newborns: Case-series. *Croat. Med. J.*, **2007**, *48*(2), 234-239.

- PMID: 17436388
- [318] Hendriks, T.; de Hoog, M.; Lequin, M.H.; Devos, A.S.; Merkus, P.J.F.M. DNase and atelectasis in non-cystic fibrosis pediatric patients. *Crit. Care*, **2005**, 9(4), R351-R356. <http://dx.doi.org/10.1186/cc3544> PMID: 16137347
- [319] Brinkmann, V.; Reichard, U.; Goosmann, C.; Fauler, B.; Uhlemann, Y.; Weiss, D.S.; Weinrauch, Y.; Zychlinsky, A. Neutrophil extracellular traps kill bacteria. *Science*, **2004**, 303(5663), 1532-1535. <http://dx.doi.org/10.1126/science.1092385> PMID: 15001782
- [320] Papayannopoulos, V.; Staab, D.; Zychlinsky, A. Neutrophil elastase enhances sputum solubilization in cystic fibrosis patients receiving DNase therapy. *PLoS One*, **2011**, 6(12), e28526. <http://dx.doi.org/10.1371/journal.pone.0028526> PMID: 22174830
- [321] Barnes, B.J.; Adrover, J.M.; Baxter-Stoltzfus, A.; Borczuk, A.; Cools-Lartigue, J.; Crawford, J.M.; Daßler-Plenker, J.; Guerci, P.; Huynh, C.; Knight, J.S.; Loda, M.; Looney, M.R.; McAllister, F.; Rayes, R.; Renaud, S.; Rousseau, S.; Salvatore, S.; Schwartz, R.E.; Spicer, J.D.; Yost, C.C.; Weber, A.; Zuo, Y.; Egeblad, M. Targeting potential drivers of COVID-19: Neutrophil extracellular traps. *J. Exp. Med.*, **2020**, 217(6), e20200652. <http://dx.doi.org/10.1084/jem.20200652> PMID: 32302401
- [322] Skovbjerg, H.; Sjöström, H.; Norén, O. Purification and characterisation of amphiphilic lactase/phlorizin hydrolase from human small intestine. *Eur. J. Biochem.*, **1981**, 114(3), 653-661. <http://dx.doi.org/10.1111/j.1432-1033.1981.tb05193.x> PMID: 6786877
- [323] Swallow, D.M. Genetics of lactase persistence and lactose intolerance. *Annu. Rev. Genet.*, **2003**, 37, 197-219. <http://dx.doi.org/10.1146/annurev.genet.37.110801.143820> PMID: 14616060
- [324] Mantei, N.; Villa, M.; Enzler, T.; Wacker, H.; Boll, W.; James, P.; Hunziker, W.; Semenza, G. Complete primary structure of human and rabbit lactase-phlorizin hydrolase: Implications for biosynthesis, membrane anchoring and evolution of the enzyme. *EMBO J.*, **1988**, 7(9), 2705-2713. <http://dx.doi.org/10.1002/j.1460-2075.1988.tb03124.x> PMID: 2460343
- [325] Fassio, F.; Facioni, M.S.; Guagnini, F. Lactose Maldigestion, Malabsorption, and Intolerance: A Comprehensive Review with a Focus on Current Management and Future Perspectives. *Nutrients*, **2018**, 10(11), E1599. <http://dx.doi.org/10.3390/nu10111599> PMID: 30388735
- [326] Leis, R.; de Castro, M.J.; de Lamas, C.; Picáns, R.; Couce, M.L. Effects of Prebiotic and Probiotic Supplementation on Lactase Deficiency and Lactose Intolerance: A Systematic Review of Controlled Trials. *Nutrients*, **2020**, 12(5), E1487. <http://dx.doi.org/10.3390/nu12051487> PMID: 32443748
- [327] Szilagyi, A.; Ishayek, N. Lactose Intolerance, Dairy Avoidance, and Treatment Options. *Nutrients*, **2018**, 10(12), E1994. <http://dx.doi.org/10.3390/nu10121994> PMID: 30558337
- [328] Portincasa, P.; Di Ciaula, A.; Vacca, M.; Montelli, R.; Wang, D.Q.; Palasciano, G. Beneficial effects of oral tilactase on patients with hypolactasia. *Eur. J. Clin. Invest.*, **2008**, 38(11), 835-844. <http://dx.doi.org/10.1111/j.1365-2362.2008.02035.x> PMID: 19021701
- [329] Ojetti, V.; Gigante, G.; Gabrielli, M.; Ainora, M.E.; Mannocci, A.; Lauritano, E.C.; Gasbarrini, G.; Gasbarrini, A. The effect of oral supplementation with *Lactobacillus reuteri* or tilactase in lactose intolerant patients: Randomized trial. *Eur. Rev. Med. Pharmacol. Sci.*, **2010**, 14(3), 163-170. PMID: 20391953
- [330] de Vrese, M.; Laue, C.; Offick, B.; Soeth, E.; Repenning, F.; Thoß, A.; Schrezenmeir, J. A combination of acid lactase from *Aspergillus oryzae* and yogurt bacteria improves lactose digestion in lactose maldigesters synergistically: A randomized, controlled, double-blind cross-over trial. *Clin. Nutr.*, **2015**, 34(3), 394-399. <http://dx.doi.org/10.1016/j.clnu.2014.06.012> PMID: 25042846
- [331] Ibba, I.; Gilli, A.; Boi, M.F.; Usai, P. Effects of exogenous lactase administration on hydrogen breath excretion and intestinal symptoms in patients presenting lactose malabsorption and intolerance. *BioMed Res. Int.*, **2014**, 2014, 680196. <http://dx.doi.org/10.1155/2014/680196> PMID: 24967391
- [332] EFSA. *EFSA J.*, **2009**, 7(9)
- [333] Jenner, J.R.; Buttle, D.J.; Dixon, A.K. Mechanism of action of intradiscal chymopapain in the treatment of sciatica: A clinical, biochemical, and radiological study. *Ann. Rheum. Dis.*, **1986**, 45(6), 441-449. <http://dx.doi.org/10.1136/ard.45.6.441> PMID: 3729572
- [334] Matsuyama, Y.; Chiba, K. Condoliase for treatment of lumbar disc herniation. *Drugs Today (Barc)*, **2019**, 55(1), 17-23. <http://dx.doi.org/10.1358/dot.2019.55.1.2899445> PMID: 30740609
- [335] Ebata, M.; Yasunobu, K.T.; Chymopapain, I. Chymopapain. I. Isolation, crystallization, and preliminary characterization. *J. Biol. Chem.*, **1962**, 237, 1086-1094. [http://dx.doi.org/10.1016/S0021-9258\(18\)60289-3](http://dx.doi.org/10.1016/S0021-9258(18)60289-3) PMID: 13888995
- [336] Maes, D.; Bouckaert, J.; Poortmans, F.; Wyns, L.; Looze, Y. Structure of chymopapain at 1.7 Å resolution. *Biochemistry*, **1996**, 35(50), 16292-16298. <http://dx.doi.org/10.1021/bi961491w> PMID: 8973203
- [337] Henschke, N.; Kuijpers, T.; Rubinstein, S.M.; van Middekoop, M.; Ostelo, R.; Verhagen, A.; Koes, B.W.; van Tulder, M.W. Injection therapy and denervation procedures for chronic low-back pain: A systematic review. *Eur. Spine J.*, **2010**, 19(9), 1425-1449. <http://dx.doi.org/10.1007/s00586-010-1411-0> PMID: 20424870
- [338] Simmons, J.W.; Upman, P.J.; Stavino, W.B. Pharmacologic and toxicologic profile of chymopapain B (Chemo-lase). *Drug Chem. Toxicol.*, **1984**, 7(3), 299-314. <http://dx.doi.org/10.3109/01480548409035110> PMID: 6376060
- [339] Cusick, J.F.; Ho, K.C.; Schamberg, J.F. Subarachnoid hemorrhage following chymopapain chemonucleolysis. Case report. *J. Neurosurg.*, **1987**, 66(5), 775-778. <http://dx.doi.org/10.3171/jns.1987.66.5.0775> PMID: 3572504
- [340] Dyck, P. Paraplegia following chemonucleolysis. A case report and discussion of neurotoxicity. *Spine*, **1985**, 10(4), 359-362. <http://dx.doi.org/10.1097/00007632-198505000-00012> PMID: 2931832
- [341] Buchman, A.; Wright, R.B.; Wichter, M.D.; Whisler, W.W.; Bosch, A. Hemorrhagic complications after the lumbar injection of chymopapain. *Neurosurgery*, **1985**, 16(2), 222-224. <http://dx.doi.org/10.1227/00006123-198502000-00017> PMID: 3883224
- [342] Ishibashi, K.; Iwai, H.; Koga, H. Chemonucleolysis with chondroitin sulfate ABC endolyase as a novel minimally in-

- vasive treatment for patients with lumbar intervertebral disc herniation. *J. Spine Surg.*, **2019**, 5(Suppl. 1), S115-S121.
<http://dx.doi.org/10.21037/jss.2019.04.24> PMID: 31380500
- [343] Hedtmann, A.; Steffen, R.; Krämer, J. Prospective comparative study of intradiscal high-dose and low-dose collagenase *Versus* chymopapain. *Spine*, **1987**, 12(4), 388-392.
<http://dx.doi.org/10.1097/00007632-198705000-00016> PMID: 3039670
- [344] Zook, B.C.; Kobrine, A.I. Effects of collagenase and chymopapain on spinal nerves and intervertebral discs of cynomolgus monkeys. *J. Neurosurg.*, **1986**, 64(3), 474-483.
<http://dx.doi.org/10.3171/jns.1986.64.3.0474> PMID: 3005529
- [345] Sussman, B.J.; Bromley, J.W.; Gomez, J.C. Injection of collagenase in the treatment of herniated lumbar disk. Initial clinical report. *JAMA*, **1981**, 245(7), 730-732.
<http://dx.doi.org/10.1001/jama.1981.03310320052026> PMID: 6257939
- [346] Huang, W.; Lunin, V.V.; Li, Y.; Suzuki, S.; Sugiura, N.; Miyazono, H.; Cygler, M. Crystal structure of *Proteus vulgaris* chondroitin sulfate ABC lyase I at 1.9 Å resolution. *J. Mol. Biol.*, **2003**, 328(3), 623-634.
[http://dx.doi.org/10.1016/S0022-2836\(03\)00345-0](http://dx.doi.org/10.1016/S0022-2836(03)00345-0) PMID: 12706721
- [347] Benito-Arenas, R.; Zarate, S.G.; Revuelta, J.; Bastida, A. Chondroitin Sulfate-Degrading Enzymes as Tools for the Development of New Pharmaceuticals. *Catalysts*, **2019**, 9(4)
<http://dx.doi.org/10.3390/catal9040322>
- [348] Hettiaratchi, M.H.; O'Meara, M.J.; O'Meara, T.R.; Pickering, A.J.; Letko-Khait, N.; Shoichet, M.S. Reengineering biocatalysts: Computational redesign of chondroitinase ABC improves efficacy and stability. *Sci. Adv.*, **2020**, 6(34), eabc6378.
<http://dx.doi.org/10.1126/sciadv.abc6378> PMID: 32875119
- [349] Chiba, K.; Matsuyama, Y.; Seo, T.; Toyama, Y. Condoliase for the Treatment of Lumbar Disc Herniation: A Randomized Controlled Trial. *Spine*, **2018**, 43(15), E869-E876.
<http://dx.doi.org/10.1097/BRS.0000000000002528> PMID: 29257028
- [350] Matsuyama, Y.; Chiba, K.; Iwata, H.; Seo, T.; Toyama, Y. A multicenter, randomized, double-blind, dose-finding study of condoliase in patients with lumbar disc herniation. *J. Neurosurg. Spine*, **2018**, 28(5), 499-511.
<http://dx.doi.org/10.3171/2017.7.SPINE161327> PMID: 29424676
- [351] Pilcher, B.K.; Wang, M.; Qin, X.J.; Parks, W.C.; Senior, R.M.; Welgus, H.G. Role of matrix metalloproteinases and their inhibition in cutaneous wound healing and allergic contact hypersensitivity. *Ann. N. Y. Acad. Sci.*, **1999**, 878, 12-24.
<http://dx.doi.org/10.1111/j.1749-6632.1999.tb07671.x> PMID: 10415717
- [352] Vogel, W.F. Collagen-receptor signaling in health and disease. *Eur. J. Dermatol.*, **2001**, 11(6), 506-514.
 PMID: 11701397
- [353] Balbin, M.; Fueyo, A.; Knäuper, V.; Pendás, A.M.; López, J.M.; Jiménez, M.G.; Murphy, G.; López-Otín, C. Collagenase 2 (MMP-8) expression in murine tissue-remodeling processes. Analysis of its potential role in postpartum involution of the uterus. *J. Biol. Chem.*, **1998**, 273(37), 23959-23968.
<http://dx.doi.org/10.1074/jbc.273.37.23959> PMID: 9727011
- [354] Duarte, A.S.; Correia, A.; Esteves, A.C. Bacterial collagenases - A review. *Crit. Rev. Microbiol.*, **2016**, 42(1), 106-126.
<http://dx.doi.org/10.3109/1040841X.2014.904270> PMID: 24754251
- [355] Gross, J.; Nagai, Y. Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. *Proc. Natl. Acad. Sci. USA*, **1965**, 54(4), 1197-1204.
<http://dx.doi.org/10.1073/pnas.54.4.1197> PMID: 4286832
- [356] Eckhard, U.; Huesgen, P.F.; Brandstetter, H.; Overall, C.M. Proteomic protease specificity profiling of clostridial collagenases reveals their intrinsic nature as dedicated degraders of collagen. *J. Proteomics*, **2014**, 100(100), 102-114.
<http://dx.doi.org/10.1016/j.jprot.2013.10.004> PMID: 24125730
- [357] Bond, M.D.; Van Wart, H.E. Purification and separation of individual collagenases of *Clostridium histolyticum* using red dye ligand chromatography. *Biochemistry*, **1984**, 23(13), 3077-3085.
<http://dx.doi.org/10.1021/bi00308a035> PMID: 6087887
- [358] Bond, M.D.; Van Wart, H.E. Characterization of the individual collagenases from *Clostridium histolyticum*. *Biochemistry*, **1984**, 23(13), 3085-3091.
<http://dx.doi.org/10.1021/bi00308a036> PMID: 6087888
- [359] Bond, M.D.; Van Wart, H.E. Relationship between the individual collagenases of *Clostridium histolyticum*: Evidence for evolution by gene duplication. *Biochemistry*, **1984**, 23(13), 3092-3099.
<http://dx.doi.org/10.1021/bi00308a037> PMID: 6087889
- [360] Worthington, K.; Worthington, V. *Worthington Enzyme Manual*, **2011**. Available from: <http://www.worthington-biochem.com/pap/default.html> Worthington Biochemical Corporation.
- [361] French, M.F.; Bhowan, A.; Van Wart, H.E. Identification of *Clostridium histolyticum* collagenase hyperreactive sites in type I, II, and III collagens: Lack of correlation with local triple helical stability. *J. Protein Chem.*, **1992**, 11(1), 83-97.
<http://dx.doi.org/10.1007/BF01025095> PMID: 1325154
- [362] Matsushita, O.; Jung, C.M.; Katayama, S.; Minami, J.; Takahashi, Y.; Okabe, A. Gene duplication and multiplicity of collagenases in *Clostridium histolyticum*. *J. Bacteriol.*, **1999**, 181(3), 923-933.
<http://dx.doi.org/10.1128/JB.181.3.923-933.1999> PMID: 9922257
- [363] Eckhard, U.; Schönauer, E.; Nüss, D.; Brandstetter, H. Structure of collagenase G reveals a chew-and-digest mechanism of bacterial collagenolysis. *Nat. Struct. Mol. Biol.*, **2011**, 18(10), 1109-1114.
<http://dx.doi.org/10.1038/nsmb.2127> PMID: 21947205
- [364] Wilson, J.J.; Matsushita, O.; Okabe, A.; Sakon, J. A bacterial collagen-binding domain with novel calcium-binding motif controls domain orientation. *EMBO J.*, **2003**, 22(8), 1743-1752.
<http://dx.doi.org/10.1093/emboj/cdg172> PMID: 12682007
- [365] Eckhard, U.; Schönauer, E.; Brandstetter, H. Structural basis for activity regulation and substrate preference of clostridial collagenases G, H, and T. *J. Biol. Chem.*, **2013**, 288(28), 20184-20194.
<http://dx.doi.org/10.1074/jbc.M112.448548> PMID: 23703618
- [366] Dhillon, S. Collagenase *Clostridium Histolyticum*: A Review in Peyronie's Disease. *Drugs*, **2015**, 75(12), 1405-1412.
<http://dx.doi.org/10.1007/s40265-015-0441-7> PMID: 26201462
- [367] Kaplan, F.T. Collagenase *clostridium histolyticum* injection for the treatment of Dupuytren's contracture. *Drugs Today (Barc)*, **2011**, 47(9), 653-667.

- <http://dx.doi.org/10.1358/dot.2011.47.9.1656502> PMID: 21971540
- [368] Ramundo, J.; Gray, M. Collagenase for enzymatic debridement: A systematic review. *J. Wound Ostomy Continence Nurs.*, **2009**, *36*(6)(Suppl.), S4-S11. <http://dx.doi.org/10.1097/WON.0b013e3181bfd83> PMID: 19918148
- [369] Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/761146s000lbl.pdf
- [370] Randhawa, K.; Shukla, C.J. Non-invasive treatment in the management of Peyronie's disease. *Ther. Adv. Urol.*, **2019**, *11*(11), 1756287218823671. <http://dx.doi.org/10.1177/1756287218823671> PMID: 30792820
- [371] Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/125338s109lbl.pdf
- [372] Available from: https://www.ema.europa.eu/en/documents/product-information/xiapex-epar-product-information_it.pdf
- [373] Grazina, R.; Teixeira, S.; Ramos, R.; Sousa, H.; Ferreira, A.; Lemos, R. Dupuytren's disease: Where do we stand? *EFORT Open Rev.*, **2019**, *4*(2), 63-69. <http://dx.doi.org/10.1302/2058-5241.4.180021> PMID: 30931150
- [374] Nayar, S.K.; Pfisterer, D.; Ingari, J.V. Collagenase Clostridium Histolyticum Injection for Dupuytren Contracture: 2-Year Follow-up. *Clin. Orthop. Surg.*, **2019**, *11*(3), 332-336. <http://dx.doi.org/10.4055/cios.2019.11.3.332> PMID: 31475055
- [375] Hurst, L.C.; Badalamente, M.A.; Hentz, V.R.; Hotchkiss, R.N.; Kaplan, F.T.; Meals, R.A.; Smith, T.M.; Rodzvilla, J. Injectable collagenase clostridium histolyticum for Dupuytren's contracture. *N. Engl. J. Med.*, **2009**, *361*(10), 968-979. <http://dx.doi.org/10.1056/NEJMoa0810866> PMID: 19726771
- [376] Gelbard, M.; Goldstein, I.; Hellstrom, W.J.; McMahon, C.G.; Smith, T.; Tursi, J.; Jones, N.; Kaufman, G.J.; Carson, C.C., III Clinical efficacy, safety and tolerability of collagenase clostridium histolyticum for the treatment of peyronie disease in 2 large double-blind, randomized, placebo controlled phase 3 studies. *J. Urol.*, **2013**, *190*(1), 199-207. <http://dx.doi.org/10.1016/j.juro.2013.01.087> PMID: 23376148
- [377] Pham, C.H.; Collier, Z.J.; Fang, M.; Howell, A.; Gillenwater, T.J. The role of collagenase ointment in acute burns: A systematic review and meta-analysis. *J. Wound Care*, **2019**, *28*(Sup2), S9-S15. <http://dx.doi.org/10.12968/jowc.2019.28.Sup2.S9>
- [378] Available from: https://www.ema.europa.eu/en/documents/product-information/nexobrid-epar-product-information_en.pdf

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