Transglutaminases as Targets for Pharmacological Inhibition

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Abstract: Transglutaminases (TGases), a family of enzymes that catalyze the formation of ε-(γ-glutamyl)lysine isopeptide linkage, play an important physiological role in hemostasis, wound healing, assembly and remodeling of the extracellular matrix, cell signaling and apoptosis. Although many members of this class of enzymes have been known for decades, their role in various physiological and pathological processes is still a subject of substantial research and debate. Convincing evidence exists that TGases are involved in formation of cytotoxic proteinatious aggregates in Alzheimer's, Huntington's and other neurodegenerative diseases. However, it is not clear if elevated levels of TGases play a causative or protective role in several of these processes. Increased or defective TGase activity is a factor in cortical cataract formation, lamellar ichtyosis and fibrosis. TGase creates epitopes for the production of autoantibodies in celiac disease and possibly other autoimmune diseases. Another TGase, Factor XIIIa, is involved in the etiology of vascular diseases. Modulation of TGase activity through its selective inhibition may have therapeutic benefit in a wide variety of diseases. This paper will examine TGases as targets for the development of new therapeutics and review the progress in discovery of selective inhibitors of these enzymes.

Keywords: Transglutaminase, Factor XIII, thrombosis, neurodegenerative disease, celiac disease, inflammation, fibrosis and inhibitor.

1. INTRODUCTION

The first transglutaminase (TGase) was discovered by Heinrich Waelsch [1-3] half a century ago. The enzyme was isolated from guinea pig liver and reported to "incorporate amines into proteins". Later work led to the recognition that the scope of reactions catalyzed by TGases is broader and includes arguably their most important physiologic function: protein cross-linking. The enzyme isolated by Waelsch's group is now referred to as TG2 (formerly tissue-TGase or t-TGase). To date, nine evolutionary related genes have been identified in humans [4], encoding nine different enzymes: transglutaminases 1 through 7 (TG1-TG7), blood coagulation factor XIII (FXIII) and devoid of catalytic activity protein epb42. The TGases are recognized by the Enzyme Commission on Nomenclature as R-glutaminyl-peptide: amine γ-glutamyltransferases (EC 2.3.2.13) and classified within the same superfamily as papain and papain-like cysteine proteases, reflecting the similarities in their catalytic mechanism.

A double-displacement mechanism has been proposed for TGases (discussed in detail in section 3). In the first step the sulfhydryl group of the active site cysteine displaces ammonia from the carboxyamide moiety of a protein/peptide bound glutamine residue forming an acyl enzyme intermediate. This intermediate then reacts with an ε-amino group of a lysyl residue of a protein or peptide leading to the formation of a covalent ε-(γ-glutamyl)lysine isoamide bond. TGases have broad specificity towards the amine-donor substrate. Therefore, they efficiently catalyze the incorporation of low molecular weight amines into proteins. In the absence of an amine, water may act as a nucleophile leading to deamidation of the protein/peptide-bound glutamine residue.

TGases are vastly distributed in various tissues and body fluids and have many diverse functions. They are generally involved in processes that make the body resistant to chemical and mechanical injury or physical degradation and in tissue remodeling and repair. Several reviews deal with TGases [5-10] and the entire February 2002 issue of *Hamostaseologie* [11] is devoted to FXIII. The reader is referred to these publications for more in depth discussion of various physiological processes in which TGases have been implicated. In this paper, those physiological roles of TGases will be highlighted which, if aberrant, lead to pathological conditions that can be ameliorated through enzyme inhibition. The progress in discovery and development of specific TGase inhibitors will be presented along with a discussion of "proof of principle", a link between the degree of TGase inhibition and the magnitude of desired physiological effect.

2. PHYSIOLOGICAL FUNCTIONS OF TRANSGLUTAMINASES

Factor XIII

Plasma factor XIII (FXIII) is the final enzyme activated in the blood coagulation cascade. It circulates in plasma as a zymogen and as such, is a tetramer composed of two identical α chains of 83.2 kDa molecular weight and two 79.7 kDa β chains. The subunits are held together by non-covalent interactions. Cellular FXIII, devoid of the β subunits, is present in platelets, erythrocytes, monocytes and different types of macrophages in most of the tissues and organs. The process of activation of plasma FXIII starts by thrombin-catalyzed cleavage of 4-kDa fragments from both α-chain amino termini. This is followed by the binding of one calcium ion per α subunit, dissociation of the β-chains

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and a final conformational rearrangement resulting in unmasking of the active site cysteine and full FXIIIa activity ("a" denotes active enzyme). Thus the main function of FXIII β chains is to serve as a transporting unit and to stabilize the hydrophobic α-subunits in the aqueous environment of plasma.

Activation of the coagulation cascade leads to the activation of thrombin which, in turn cleaves N-terminal fibrinopeptides A and B from fibrinogen producing fibrin [12]. The cleavage of fibrinopeptides leads to unmasking of hydrophobic surfaces on fibrin. This allows the fibrin chains to organize themselves into highly ordered fibrin clot. The processes of FXIII and fibrinogen activation are greatly accelerated in the tertiary complex between fibrin, thrombin and FXIII [13-18]. Fibrin is therefore a substrate for FXIIIa and a cofactor for its activation. This has important implications for hemostasis: FXIII is not activated until sufficient amount of fibrin is generated.

Clinical symptoms observed in FXIII-deficient patients [19-21] indicate that the enzyme is physiologically important in three major areas: hemostasis, wound healing and maintaining pregnancy. The main function of plasma FXIIIa in hemostasis is to stabilize the fibrin clot formed as the blood coagulation cascade is activated in response to internal or external injury of a blood vessel. This is achieved by cross-linking of fibrin strands and a covalent attachment of hemostatic factors to the fibrin network. Cross-linking of fibrin changes the viscoelastic properties of the clot [22-26] and contributes to its increased stability against fibrinolytic enzymes [27-32]. Numerous *in vitro* studies demonstrated that plasma clots formed in the presence of FXIIIa inhibitors are characterized by increased susceptibility to fibrinolysis [33-36].

FXIIIa catalyses several processes during the formation of plasma clot. Following fibrinogen activation by thrombin, FXIIIa cross-links the γ-chains of fibrin to form γ-dimers and the α-chains to form α-polymers (for reviews on the mechanism of fibrin clot assembly see [37-40]). The formation of γ-dimers is completed within 5 to 10 minutes after clot formation and involves only one donor and one acceptor site on γ-chains [41, 42]. The polymerization of the α-chains is much slower and involves several Gln and Lys residues [43-47]. Still a slower process is the formation of γ-trimers, γ-tetramers [48-50] and small amount of linkages between γ-oligomers and α-polymers [48, 49] resulting in intricate cross-linked fibrin network. These slow processes are postulated to result in increased resistance of aged thrombi to fibrinolysis [32, 51]. Factor XIIIa also very efficiently catalyses the covalent incorporation of α_2-antiplasmin, a potent inhibitor of plasmin, into the fibrin clot [52-54]. The presence of cross-linked α_2-antiplasmin significantly increases the resistance of the clot to degradation by plasmin [55-57].

The individual contribution of various FXIIIa catalyzed reactions to the resistance of mature plasma clot to fibrinolytic agents has been a subject of a long debate. Specific inhibitors of Factor XIIIa played an important role in elucidating the connection between FXIIIa-catalyzed reactions and fibrinolytic resistance of plasma clots first *in vitro* and later *in vivo*. It is now generally believed that protection of newly formed thrombi against the fibrinolytic system is conferred mostly by α_2-antiplasmin covalently linked to fibrin [56, 58].

Plasma clots formed in arteries contain a large number of platelets [33]. Experimental [59] and clinical [60-62] evidence indicates that platelets play an important role in clot stabilization against fibrinolysis. The enhanced resistance of platelet-rich clots to degradation by plasmin is partially due to platelet FXIII [63].

Another role of FXIIIa is to anchor the clot to the site of injury by cross-linking fibrin to adhesive glycoproteins (reviewed in [8]) such as fibronectin and thrombospondin. The enzyme can also cross-link monomeric von Willebrand factor, a plasma glycoprotein involved in facilitating initial adhesion of platelets to vascular subendothelium. FXIIIa catalyzes the incorporation of amines into von Willebrand factor and its cross-linking to fibrin and monomeric collagen. During the assembly of the extracellular matrix (ECM), FXIIIa cross-links fibronectin into high molecular weight polymers thus facilitating cell adhesion to the ECM. Other adhesive ECM proteins, such as vitronectin, osteopontin and elastin are also substrates for FXIIIa.

TG2

In contrast to other TGases, all of which specialize in cross-linking of proteins in specific biological processes, TG2 is still an enigmatic, multifunctional enzyme with many possible, but not yet clearly understood, roles in various tissues [6, 64-66] and many diverse physiological events. It is largely due to the fact that, as the only member of this gene family, TG2 displays both Ca^{2+}-dependent transamidase activity and GTP-dependent signal transducing activity [67]. Furthermore, in addition to its several cellular functions both in the cytosol and in association with the membrane, the enzyme can be externalized from cells and interact with the ECM proteins. As mentioned earlier, the Ca^{2+}-dependent transamidase activity is not limited to protein cross-linking, but can also involve hydrolysis of isoamide bonds, site-specific incorporation of amines and deamidation of glutamine in proteins and peptides.

In its GTP/GDP bound form, TG2 cannot catalyze transamidation. Calcium ions act as a switch between the guanine triphosphatase activity and the transamidase activity of TG2. The role of TG2 as a G-protein (historically referred to as the G_h protein) in signal transduction is clearly established [68, 69]: G_h/TG2 stimulates phospholipase C mediated production of inositol phosphate in response to agonist activation of α_{1B}- and α_{1D}-adrenergic receptors [67, 70] TP A thromboxane A_2 [71] and oxytocin receptors [72].

The involvement of TG2 in the process of apoptosis has been recognized for a long time. The enzyme is induced in cells undergoing apoptosis *in vivo* [73] and its overexpression is postulated to prime the cells for suicide [74]. During later stages of apoptosis, the increase of the intracellular calcium concentration "turns on" the transamidase activity of TG2 which results in massive cross-linking of intracellular proteins. The formation of insoluble protein polymers prior to phagocytosis prevents the release of harmful intracellular components that could otherwise trigger inflammatory or autoimmune responses [75].

TG2 plays an important role in paracellular processes such as assembly, stabilization and remodeling of the ECM by cross-linking of fibronectin, fibrinogen, fibrin, von Willebrand factor, collagen V and other components of the ECM (for review see [6]). Increasing evidence suggests that cross-linking of cell surface-associated fibronectin promotes cell migration and adhesion to the matrix [76-78]. TG2 binds to fibronectin with high affinity [78-80]. Furthermore, the enzyme modulates the biological activity of several ECM signaling proteins [81-86], including interleukin-2, midkine and latent transforming growth factor β1 (TGF β1) [84].

TG2 is directly involved in wound healing and angiogenesis [87]. The enzyme has been implicated in cellular maturation and differentiation processes [88], bone ossification [79] and cross-linking of histons in the nucleus [89].

Equipped with a powerful catalytic apparatus, TG2 catalyzes a variety of biochemical reactions with many substrates. Future research is needed to clarify which of the processes that TG2 can potentially catalyze or mediate are critical for the survival of the cell, which are redundant and which are merely *in vitro* discovered possibilities without any *in vivo* significance. It is particularly surprising that the homozygous deletion of TG2 in mouse leads to viable, normal size animals [90, 91] with no obvious alterations in apoptosis, the structure of ECM or the heart function. Certain alterations expected on the basis of proposed physiological functions of TG2, for example impaired wound healing, decreased adherence of primary fibroblasts and impaired clearance of apoptotic cells by phagocytosis, manifest themselves in TG2-/- mice under certain pathological or stress conditions. No TG2 deficiency in man has been found yet.

Epidermal TGases

At least three TGases, membrane-associated TG1 and cytosolic TG3 and TG5, are involved in formation of cornified cell envelope (CE) [92-94], a 15-nm protective layer of cross-linked proteins on the inner surface of the cell periphery in the epidermis. The cytosolic TGases, in particular TG3, are involved in the cross-linking of several soluble CE proteins into oligomers. These are then translocated to the cell membrane and incorporated into growing CE structure by membrane-associated TG1 [93, 95]. A congenital keratinization disorder, lamellar ichtyosis, has been linked to mutations in the TG1 gene. The disorder is characterized by thickened epidermis and large fish-like scales with high risk of sepsis and dehydration. Mice lacking the TG1 gene show a phenotype similar to the patients suffering from lamellar ichtyosis [96].

3. STRUCTURE AND CATALYTIC MECHANISM OF TRANSGLUTAMINASES

The zymogen form of recombinant cellular FXIII was the first TGase for which a three-dimensional structure has been determined [97, 98]. The detailed structural information obtained through crystallographic studies provided evidence that largely confirmed earlier views on enzyme mechanism [99]. It has also revealed several interesting structure-function correlations in the enzyme activation and the catalytic processes.

Fig. (1). Structure of FXIIIa α subunit. Enzyme structural domains are shown in different colors: activation peptide (pink), the beta sandwich domain (blue), the catalytic core (red) and the beta barrels 1 and 2 (white). The catalytic triad residues, Cys314, His373 and Asp396, are shown in green. Protein structure was obtained from RCSB PDB [100], http://www.pdb.org/. PDB ID: 1F13 [101]

Cellular FXIII is a homodimer (α_2). The FXIII α subunit is well-organized into four sequentially folded domains, as shown in (Fig. **1**). The N-terminal activation peptide (residues 1-37), which does not have any distinct tertiary structure, is followed by the beta sandwich domain (residues 38-183), the catalytic core (residues 184-515) and beta barrels 1 (residues 516-627) and 2 (residues 628-730). The catalytic triad, Cys314-His373-Asp396, and the overall organization of the catalytic domain are very similar to Cys-His-Asn triad and the catalytic core of cysteine proteinases from the papain family. These striking similarities strongly support an earlier proposed [99] analogy between their catalytic mechanisms: that of FXIIIa being essentially a reversal of the proteolytic mechanism of cysteine proteinases. In the case of FXIIIa and other TGases, however, water has to be excluded from the enzyme active site. An amino group of Trp 279 residue and the backbone NH of Cys314 have been proposed to stabilize the oxyanion intermediate in FXIIIa [102], a role analogous to that of highly conserved glutamine and backbone amide of the active site cysteine, known as "oxyanion hole" in cysteine proteinases.

The proposed transamidation mechanism [102, 103] involving a cysteine proteinase-like catalytic triad and an oxyanion hole is depicted in (Fig. **2**). In the first step, the active site cysteine reacts with γ-carboxyamide of glutamine residue forming a tetrahedral oxyanion intermediate that

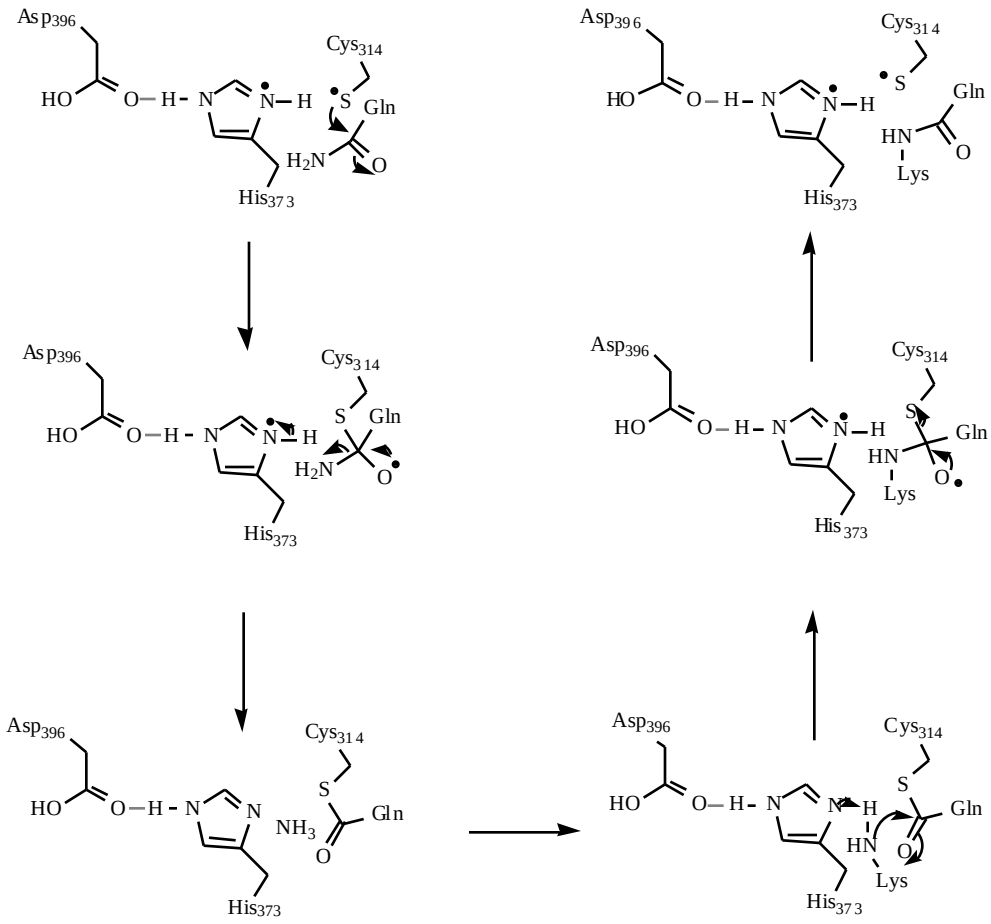


Fig. (2). Proposed catalytic mechanism for TGases [102]. Residue numbers are given for FXIIIa.

collapses giving a thioacyl enzyme and releasing ammonia. The ε-amino group of lysine then attacks the carbonyl group of the thioacyl enzyme intermediate yielding a second oxyanion intermediate that finally collapses to form the isopeptide bond and regenerate the active enzyme.

A large change in protein conformation has to take place upon enzyme activation to open access to the active site and expose the active site cysteine. The crystal structure of zymogen revealed that Tyr560 residue from the first beta barrel domain is hydrogen bonded to active site Cys314 and has to be removed during the activation process. Furthermore, the access to the active site is blocked by the activation peptide from the other α subunit of the α_2 dimer. Interestingly, this peptide remains associated with the enzyme even after proteolytic cleavage by thrombin and the overall protein conformation is very similar to that of intact zymogen [104]. The crystal structures of FXIII crystallized in the presence of calcium and strontium [98] allowed the localization of ion binding sites and revealed no structural changes other than minor local rearrangements of the amino acid residues directly involved in metal binding. Since neither binding of calcium alone nor the proteolysis of the activation peptide resulted in the switch to the active conformation of the enzyme, it was postulated that substrate binding is necessary to initiate this process.

Hilgenfeld and coworkers [101] used their high resolution X-ray data collected for FXIII zymogen to propose a possible mechanism for the activation process. In the view of the authors, dissociation of the activation peptide and partial separation of the catalytic domain and the first beta barrel domain, both necessary for the exposure of the active site, are energetically demanding. This energy cannot be gained by binding small peptides known to be good FXIIIa substrates. It may however be provided by isomerization of two high energy non-proline *cis* amide bonds, found near the active site of the enzyme, to their *trans* conformation. This event may then trigger the conformational switch. The crystal structure of FXIIIa in its active conformation has not been reported.

A three-dimensional structure of human TG2 complexed with GDP was solved [105]. The general domain structure of this enzyme is similar to that of FXIII α subunit: sequentially folded N-terminal beta sandwich, followed by catalytic domain and two beta barrel domains. The architecture of the transamidation active site involving the catalytic triad Cys277-His335-Asp358 is very similar to that of FXIII. The guanine nucleotide binding site is located in a cleft between the catalytic and the first beta barrel domains. As expected from lack of sequence homology, the GTP/GDP binding site of TG2 differs markedly from that of large heterodimeric G proteins and small Ras-related G-proteins. The details of TG2 structure revealed by the crystallographic study provide clues about the mechanism of regulation of TG2 transamidation activity by guanine nucleotide binding. In the GDP-bound form, the access to the transamidation site is blocked by two loops connecting the beta strands of

the first beta barrel domain. As in FXIII, the Cys277 (equivalent to Cys314 in FXIII) is hydrogen bonded to a highly conserved Tyr516 (equivalent to Tyr560 in FXIII). Both, the beta barrel 1 and Tyr516 associated with it, must be removed to allow access of the substrates to the transamidation active site. GDP engages the first and the last beta strands of the first beta barrel domain and stabilizes the loops that occlude the access to the transamidase catalytic domain. This mechanism is likely to account for the observation that guanine nucleotide binding inhibits transamidation activity [106, 107].

Three-dimensional structures of recombinant human TG3 in its zymogen and activated forms were reported recently [108]. The crystal structures and supporting biochemical studies revealed the presence of one calcium tightly bound to the catalytic domain of the zymogen. Two additional calcium ions, required for enzyme activity, bind to the active site domain after proteolytic activation of the enzyme and significantly stabilize the tertiary structure. The zymogen and the activated forms of TG3 are organized in the same way as FXIII, including the catalytic triad and the presence of two non-proline *cis* peptide bonds.

4. TRANSGLUTAMINASES IN DISEASE

TGases have been implicated in many pathological processes. The possible role of this family of enzymes in the ethnology of various diseases has been discussed in several excellent reviews [10, 109-111]. The reader is also referred to the entire issue of *Neurochemistry International* [112] devoted to the role of TGases in neurodegenerative diseases.

Thrombotic Diseases

Recent studies have shown a protective effect of a common polymorphism in FXIII α subunit gene (Val34Leu) against myocardial infarction (MI) [113, 113, 114], ischaemic stroke [115] and deep vein thrombosis [116]. These studies provided for the first time a direct link between Factor XIII and thrombotic diseases and resulted in renewed interest in this enzyme [117]. It is proposed that Val34Leu mutation leads to altered interaction of FXIII with thrombin and to excessive activation of the mutant enzyme [118, 119]. This may result in premature depletion of FXIII(Val34Leu) from circulation and cause the antithrombotic effect.

Selective inhibition of FXIIIa may constitute a new way of controlling thrombosis by facilitating endogenous or pharmacologically induced fibrinolysis [120]. Of particular interest is application of FXIIIa inhibitors as adjunctive agents to currently used thrombolytic agents in acute MI. The primary goal of current treatment for acute MI involves rapid and complete dissolution of the occluding thrombus and the restoration of blood flow (reperfusion). Thrombolytic agents, such as streptokinase, anistreplase (APSAC), alteplase (t-Pa, activase), reteplase (r-Pa) and tenecteplase (TNK-tPA), are the mainstay of pharmacological treatment for acute MI. These drugs work by activating plasminogen to plasmin, which then degrades fibrin clot thus reopening the occluded artery. Rapid restoration of blood flow is the key to preserving myocardium and improving survival [121]. Despite significant improvement in the treatment of acute MI introduced with the use of thrombolytic agents, there are several limitations to this approach. The GUSTO [121] international randomized clinical trial comparing various thrombolytic strategies demonstrated that even with rapid initiation of thrombolytic therapy and optimal use of approved adjunctive agents, aspirin and heparin, a significant number of patients do not achieve reperfusion. Large scale clinical trials [122-125] have shown that typically, after 90 minutes of thrombolytic therapy, reperfusion is only established in approximately 30 to 60 % of patients. Another limitation of the thrombolytic therapy is re-formation of an occlusive clot following incomplete thrombolysis. Reocclusion of the infarct-related artery within the first 24 hours occurs in 5-15% of patients [126] and leads to two- to threefold increase in mortality.

Thrombolytic therapy is associated with increased risk of bleeding complications, particularly intracranial hemorrhage (ICH). The risk of ICH is higher in patients receiving clot-specific drugs, such as alteplase, reteplase and tenecteplase, than in those who receive streptokinase [121]. A unique feature of FXIIIa inhibition is that it does not result in a prolongation of plasma clotting time [34-36, 127, 128]. Therefore, agents targeting FXIIIa are expected to be generally safer than the drugs targeting any other enzyme in the coagulation cascade.

Neurodegenerative Diseases

TG2 is expressed in neurons *in vivo* [129] and TGase activity has been detected in many neural tissues including brain, spinal cord, peripheral nerve and superior cervical ganglia [130, 131]. The role of TG2 in neuronal function is not fully understood. In addition to protein cross-linking in apoptosis [132], the enzyme may be involved in neural development and regeneration (for reviews see [131, 133]).

Recent advances in research on various neurodegenerative diseases led to the discovery of a shared feature in many of these disorders: the accumulation of highly insoluble, polymeric aggregates of disease-specific proteins in the brain. Accumulation of these aggregates in the neural cells leads to impairment of cellular function. It has been suggested that the deregulation of neuronal TG2 activity and uncontrolled cross-linking of the disease specific protein aggregates may lead to pathogenesis of Alzheimer's disease, and several $(CAG)_n/Q_n$ expansion disorders including Huntington's disease.

Alzheimer's disease (AD), the most common age-related neurodegeneratve disease, is associated with progressive damage of neuronal circuits in certain regions of the brain leading to memory loss, impaired thinking and behavior (for a review see [131]). The main pathophysiological feature of AD is the presence of extracellular neuritic senile plaques and intraneuronal neurofibrillary tangles (NFT) that are highly insoluble and resistant to proteolysis. The latter are composed primarily of paired helical filaments (PHF). The main component of PHF is the hyperphosphorylated form of the microtubule associated protein *tau*. Several observations support the hypothesis that TGase catalyzed cross-linking of *tau* protein plays a role in the neuropathology of AD. TG2 and several other TGases are expressed in the neuronal membrane, cytosol and the nucleus [134, 135]. Expression of these enzymes and their cross-linking activity is increased

in the AD brains [134, 136]. Buffer soluble *tau* protein is an excellent substrate for TGase [137], suggesting that the cross-linking might facilitate its aggregation. Furthermore, increased number of intraneuronal TGase-catalysed cross-links were found in an AD brain compared to brains without neurofibrillary pathology and the ε-(γ-glutamyl)lysine bonds co-localized with AD NFT [138]. Importantly, it was demonstrated that the cross-linking of PHF *tau* protein precedes the formation of senile plaques and NFT indicating that the cross-linking of *tau* is an early phenomenon and not just a secondary effect that occurs after the amyloid plaques and the NFT have been formed in the brain. It however remains controversial whether the cross-linking of *tau* protein by TGase is one of the factors contributing to the etiology of AD or it plays a protective role by removing the toxic protein from the aqueous phase.

Huntington's disease (HD), a neurological disease that inevitably leads to death in affected individuals, is an example of polyglutamine expansion disorders ($(CAG)_n/Q_n$-expansion disorders) which include seven other neurodegenarative diseases [111, 139-142]. The pathological hallmark of HD is the aggregation of polymerized huntingtin in neuronal intranuclear inclusions (NIIs) and dystrophic neurites (DNs) in the cortex and striatum [110]. Huntingtin contains a stretch of several glutamine residues near its N-terminus. HD develops when the length of the polyglutamine domain exceeds 36 residues. As in the case of cross-linked *tau* protein in AD, it is not known whether huntingtin aggregates are destructive for neurons or they are simply a result of body's protective reaction to remove a noxious agent from solution. Indeed, experimental evidence exists that the huntingtin aggregates are not required for the initiation of the cell death and that the mutated huntingtin may be more toxic if not sequestered into an aggregate (for a review see [133]).

It is postulated that two types of processes are involved in huntingtin aggregation: formation of "polar zippers" between main-chain and side-chain amide groups leading to highly insoluble β-pleated sheet structures [143] and TGase catalyzed cross-linking of abnormally expanded polyglutamine domain [144-146]. However, there is still not enough convincing evidence for the formation of amyloid type structures, expected from ordered β-pleated sheets, *in vivo* in HD brain. On the other hand, TG2 cross-links huntingtin *in vitro* [146-148] and the rate constant for the reaction increases when the number of glutamine repeats is larger than 36 [147]. TG2 protein levels and TGase activity is increased in brains affected with HD [129, 147]. Immunohistochemical studies demonstrated the existence of ε-(γ-glutamyl)lysine bonds co-localized with huntingtin protein and TG2 in the nuclei in the frontal cortex in HD brains but not in control disease-free brains [149].

Inhibition of TG2 with cystamine and monodansyl cadaverine was demonstrated to significantly reduce the formation of protein aggregates in several cellular models of $(CAG)_n/Q_n$ expansion disorders [146, 148, 150]. The hypothesis that the inhibition of TG2 might be beneficial in HD was tested in transgenic R6/2 mice expressing exon 1 of huntingtin with an expanded polyglutamine region [151]. Cystamine, a competitive inhibitor of TG2, was administered intraperitoneally to the animals and the progress of the disease was observed. Cystamine or, as pointed out by Cooper [111], its reduced form cysteamine entered the brain and inhibited TGase activity. Treatment extended survival, reduced tremor and abnormal movements in animals with HD-like disease. The treatment, however, did not change the appearance or frequency of NIIs. Unexpectedly, cystamine treatment increased the transcription of one of the two neuroprotective genes shown to be protective against polyglutamine toxicity in *Drosophila*, *dnaj*. Although at this stage one cannot rule out an indirect systemic effect of cystamine on the progress of HD, the results suggest that inhibition of TGase may provide a new way of treatment of this disease and possibly other $(CAG)_n/Q_n$ expansion disorders. The presence of insoluble protein aggregates has now been reported in almost all polyglutamine repeat diseases suggesting a similar pathogenic mechanism. Possible involvement of TGase in etiology of the associated diseases has also been postulated.

Celiac Disease

Celiac disease (CD) is an autoimmune disorder, caused by sensitivity to gliadin, component of wheat gluten. It results from an inappropriate T-cell mediated response against ingested gluten and peptides resulting from its breakdown. Clinically, CD manifests itself as a chronic inflammation of the small intestine. Many factors, including genetic, immunological and environmental, may contribute to this disorder and produce a wide range of clinical manifestations.

The discovery that TG2 is one of the main antigens in CD [152] has accelerated research on the pathogenesis of this disease. High levels of TG2 were found in the intestines of CD patients compared to the control population [153]. It was demonstrated that TG2 deamidates glutamine residues in gliadin *in vitro* creating negatively charged glutamic acid residues. These become neoepitopes and stimulate the proliferation of gliadin-specific T-cells [154, 155]. TG2 also cross-links itself to gliadin and its fragments *in vitro* and stimulates production of antibodies against gliadin, TG2 and the cross-linked proteins [154, 155]. A similar mechanism has been postulated *in vivo*, but it is still unclear whether any of these processes, the deamidation and the cross-linking of TG2 to gliadin, operate *in vivo* and how they contribute to the pathogenesis of CD. Similarly, TGase may contribute to development of other autoimmune disorders in which either anti-TG2 antibodies have been detected or the participation of TGase substrates has been documented [156].

Cataract Formation

Development of cortical cataract, progressive opacification of the eye, may lead to blindness. Cataracts result from aberrant cross-linking of predominantly β-crystallin in the cornea. TGase is postulated to play an important role in this process. Increased TGase activity might be a consequence of well-documented elevation of intracellular Ca^{2+} in the cataracts [157]. As mentioned before, intracellular TGase activity increases at elevated Ca^{2+} concentration [158]. Furthermore, treatment of rabbit eye with calcium ionophore, ionomycin, led to the formation of

β-crystallin dimers croslinked by ε-(γ-glutamyl)lysine bonds characteristic of TGase activity [159, 160]. Similar isopeptide links were found in the high molecular weight polymeric proteins in human cataracts [161].

Fibrosis and Inflammation

Deposition of ECM and biosynthesis and accumulation of TG2 is a part of a healthy response of an organism to tissue injury induced by inflammation, mechanical injury or tumor growth. It is postulated however, that excessive cross-linking of ECM in several pathological processes leads to increased resistance of these structures to breakdown and clearance. This, in turn, leads to the manifestation of these pathological conditions. Numerous major protein components of ECM are TGase substrates [6, 156] including fibronectin, fibrinogen, osteonectin, osteopontin, collagens, vitronectin, collagen-tailed acetylcholinesterase, elafin and plasminogen activator inhibitor 2.

Excessive fibrotic tissue accumulated in renal interstitial fibrosis, liver cirrhosis, parasitic liver fibrosis and pulmonary fibrosis [162-165] was found to be highly cross-linked by TG2. This enzyme may be even more directly involved in the regulation of matrix synthesis by modulating the activation of latent TGF-β [83, 84, 166].

Atherosclerotic plaques contain increased levels of TG2 and ε(γ-glutamyl)lysine linkages characteristic of this enzyme's activity [167, 168]. This is not surprising, since TG2 is constitutively expressed at a high level in endothelial and smooth muscle cells [64, 169] and, together with plasma FXIIIa, is involved in stabilization of fibrin clot and endothelial extracellular matrix after cell wall injury [87, 170].

It has been demonstrated that TG2 introduces an intramolecular cross-link into phospholipase A_2 (PLA_2) *in vitro* and thus induces a conformational change leading to the dimerization of the enzyme and a 10-fold increase in its specific activity [171]. *In vitro* studies demonstrated that TG2 also catalyses the incorporation of naturally occurring polyamines such as putrescine, spermidine and spermine, into PLA_2 leading to threefold increase of PLA_2 activity [172]. PLA_2 is a key enzyme in the regulation of leukotriene and prostaglandin biosynthesis in lipoxygenase and cycloxygenase pathways, respectively. This enzyme has been linked to the initiation and propagation of several inflammatory diseases and, for a long time now, has been a target for pharmacological inhibition. Thus the discovery that following an influx of calcium, activation of TG2 can potentially increase PLA_2 activity *in vivo* led to the hypothesis that simultaneous inhibition of both PLA_2 and TG2 may lead to superior anti-inflammatory therapeutics. Sohn [173] has synthesized several peptides comprising both TG2 and PLA_2 inhibitory sequences. The TG2 inhibitory sequence was modeled after elafin [174, 175], a known substrate for this enzyme and the PLA_2 peptide inhibitory sequence was designed on the basis of similarities between several anti-inflammatory proteins, presumably inhibitors of PLA_2, induced by steroidal anti-inflammatory drugs. The novel peptides inhibited both enzymes *in vitro* and *in vivo*. They were also effective in a guinea pig model of allergic conjunctivitis [173]. Based on clinical inflammation scores, one of the chimeric peptides was as potent as topical steroid or antihistamine drops. The anti-inflammatory activity correlated well with *in vitro* inhibitory potency against PLA_2 and TGase. The inhibition of these two enzymes was demonstrated in tissue extracts form treated animals.

5. PROGRESS IN INHIBITOR DESIGN

Low Molecular Weight Inhibitors of TGases

The elucidation of the mechanism of fibrin cross-linking catalyzed by FXIIIa led Lorand [176, 177] to the proposal that certain primary amines that can serve as substrates (pseudo-donors) for this enzyme will become incorporated into fibrin, effectively inhibiting its cross-linking [127]. Similarly, low molecular weight glutamine substrates for FXIIIa may serve as pseudo-acceptor sites [178-180] and reduce the number of fibrin cross-links. Structural requirements for amine pseudo-substrate inhibitors of FXIIIa were subsequently determined. To increase the potency and the selectivity of amine inhibitors of FXIIIa, structure-activity studies were undertaken to find compounds displaying affinity for the enzyme-binding site [127, 177, 181]. This led to the discovery that monotosyl- (**1)** and monodansylcadaverine (**2)**, shown in (Fig. **3**), and their

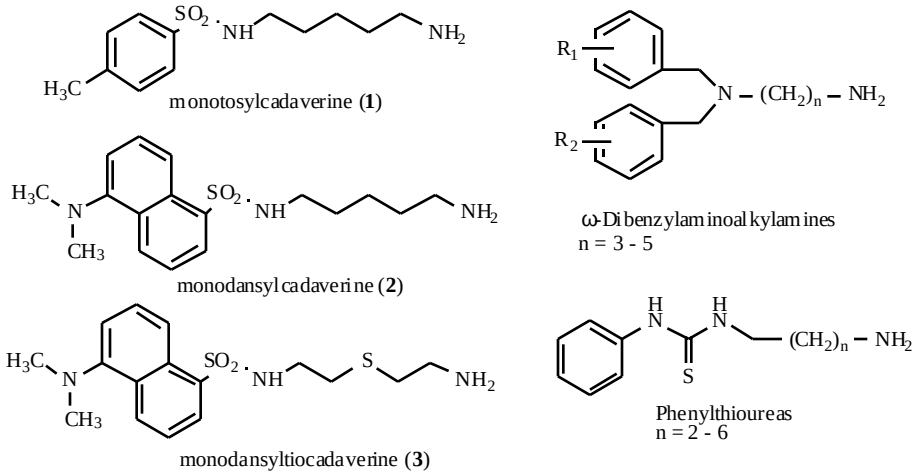


Fig. (3). Amine pseudo-substrate inhibitors of FXIIIa.

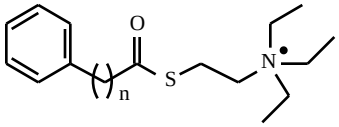
analogs are excellent pseudo-substrates for FXIIIa and therefore efficiently prevent fibrin cross-linking. The refinement of the structure-activity relationships in a series of monodansyl derivatives of alkylamines led to monodansylthiocadaverine **3**, which was six times more potent than monodansylcadaverine. These compounds became the first probes to determine whether inhibition of fibrin cross-linking facilitates *in vitro* and *in vivo* fibrinolysis. Moreover, since these compounds contain a fluorescent "reporting group" they were used to label specific glutamine residues on various proteins that are substrates for TGases [182-185]. Monodansylcadaverine and its derivatives however, have serious disadvantages that prevented their development into pharmaceuticals. They are only effective at high (millimolar) concentrations at which they inhibit other enzymes and interfere with cell attachment.

Hoffman and coworkers [186] developed ω-dibenzylaminoalkylamines, of general structure shown in (Fig. **3**), as FXIIIa inhibitors. These compounds, initially intended as alkylating agents, were demonstrated to inhibit FXIIIa acting as potent pseudo-substrate amine donors.

Specificity of TG2 for amine substrates was also investigated [99, 187]. As in the case of FXIIIa, the hydrophobic aryl moieties of pseudo-substrates, preferentially bearing electron-withdrawing substituents, are probably anchored to a specific site on the enzyme. The function of the alkyl chain is to provide a spacer between that site and the carbonyl group of a thiol ester intermediate with which the amino group is to react.

Further improvement was introduced by Lee and coworkers [188] who used a series of phenylthiourea derivatives of α,ω-diaminoalkanes, shown in (Fig. **3**), as pseudo-substrates and competitive inhibitors of both TG2 and Factor XIIIa. The most potent inhibitors of the series had K_is close to that of monodansyl cadaverine and had the advantage of being less toxic in mice. However, the development of amine pseudo-substrates for internal use was largely hindered due to their low potency, lack of enzyme selectivity and the resulting toxicity. One possible way of circumventing the toxicity is to avoid the systemic exposure by topical application. Putrescine (1,4-diamiobutane) formulated for topical application under the name of Fibrostat^{TM} is currently undergoing clinical evaluation for the prevention of hypertrophic scarring during abnormal wound healing [189].

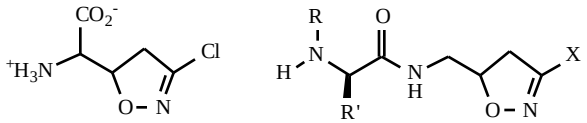
Thiol esters of carboxylic acids of general structure shown in (Fig. **4**), were also used as pseudo-acceptors [178]. Structure activity studies led to FXIIIa inhibitors **4** and **5** which were as potent as monodansyl cadaverine in a fibrin cross-linking assay system [179].



n = 2; 2-benzyldiethylammonioethyl 3-phenylthiolpropionate (**4**)
n = 3; 2-benzyldiethylammonioethyl 4-phenylthiolbutyrate (**5**)

Fig. (4). Thioester pseudo-substrate inhibitors of FXIIIa.

Irreversible inhibition (inactivation) by covalent modification of the active site cysteine of TGase is another approach to enzyme inhibition. Several nonspecific thiol-reactive reagents were demonstrated to inactivate TG2 and FXIIIa including iodoacetamide [190, 191], α-bromo-3-hydroxy-3 nitroacetophenone [192] and alkyl isocyanates [193]. Several α-halogenmethyl carbonyl compounds designed on a scaffold of amine pseudo-substrates were shown to inactivate FXIIIa in crude plasma fraction from bovine blood; their structure-activity profile confirmed previously learned lessons about the enzyme active site architecture [194]. However, high intrinsic reactivity of these compounds towards thiols and thiol-dependent enzymes is at the root of their toxicity and lack of potential therapeutic utility.



Acivicin (**6**)

R = Cbz, R' = $PhCH_2$, X = Br (**7**)
R = Cbz, R' = $PhCH_2$, X = Cl (**8**)

Fig. (5). Structure of activicin and 3-halo-4,5-dihydroisoxazoles.

In an effort to develop more selective, mechanism based inactivators of TGases, the scientists at Syntex Research introduced 3-halo-4,5-dihydroisoxazoles [195, 196] of general structure depicted in (Fig. **5**). The idea of using this small heterocycle as a scaffold for designing TGase inhibitors came from the discovery that a natural product and a glutamine antagonist, acivicin (**6**), inactivated anthranilate synthase, another cysteine-dependent enzyme, by modifying its active site cysteine. Indeed the active site cysteine thiol of guinea pig TG2 reacts with peptidyl halodihydroisoxazoles by displacing the halide [197]. The resulting stable imino thioether enzyme adduct is unable to participate in the second reaction with the ε-amino group of lysine residue. The proposed mechanism for inactivation resembles the enzyme catalytic mechanism. The dihydroisoxazole ring is activated towards nucleophilic attack by a putative proton donor on the enzyme. The resulting oxoanion intermediate collapses with the expulsion of the halide leaving group and the formation of a stable imino thioether. The identity of imino thioether enzyme adduct was confirmed by ^{13}C NMR study [197]. The second order rate constants for the inactivation of bovine epidermal TGase by **7** and **8** were 897 and 223 $M^{-1}s^{-1}$, respectively, reflecting the leaving group ability. As expected, the 3-methyl derivative was inactive. A series of 3-substituted 4,5-dihydroisoxazoles was examined for inhibition of the ionophore-induced formation of cross-linked envelopes by human malignant SCC-9 keratinocytes [196]. The compounds exhibited potency reflecting their capacity to inhibit purified bovine epidermal TGase. *S*-stereoisomers at C5 were much more active than their *R*-counterparts in both systems.

The scientists at Merck Research Laboratories took another approach to mechanism-based inactivation. They developed a series of 2-[(2-oxopropyl)thio]imidazolium derivatives of general structure depicted in (Fig. **6**) for selective inactivation of FXIIIa. In these compounds the halo leaving group of the halomethyl carbonyl compounds was replaced with a poorer leaving group, effectively limiting their intrinsic reactivity. In addition, the thioimidazolium

leaving group was designed to allow interaction with FXIIIa binding site to increase the potency and the enzyme selectivity of the inactivators. The thioacetonyl heterocycles inactivated FXIIIa and human erythrocyte TGase with second order rate constants ranging from 0.3×10^4 to 6.3×10^4 $M^{-1}s^{-1}$. These rates were 5 to 6 orders of magnitude higher than the second order rate constants for their reaction with glutathione. The mechanism of FXIIIa inactivation was investigated by the use of radiolabelled compounds and the NMR technique. It was demonstrated that FXIIIa was inactivated by acetonylation of the active site cysteinyl residue. Although the dissociation constant of the enzyme-inhibitor complexes could not be measured for these compounds, due to their high inactivation rate constants, their less reactive non-quaternary analogs were demonstrated to form a reversible complex with the enzyme before the inactivation step. Thus 2-[(2-oxopropyl)thio]imidazolium derivatives were not merely "non-specific thiol-trapping agents" but demonstrated certain degree of enzyme active-site recognition. Consequently, they did not inhibit or inactivate other thiol reagent-sensitive enzymes such as papain, calpain, fatty acid synthase, HMG CoA synthase and HMG CoA reductase at up to 1 mM concentration. 2-[(2-oxopropyl)thio]imidazolium derivatives effectively inhibited fibrin cross-linking and the cross-linking of α_2-antiplasmin to fibrin *in vitro*. The most potent inactivator of the series, 2-(1-acetonylthio)-5-methylthiazolo[2,3]-1,3,4-thiadiazolium perchlorate (**10**) at 20 μM concentration inhibited 50-60 % of γ-chain dimerization and 95% of α_2-antiplasmin incorporation into fibrin. Plasma clots produced *in vitro* in the presence of low μM concentrations of **10** lysed several times faster and more completely in response to t-Pa [34].

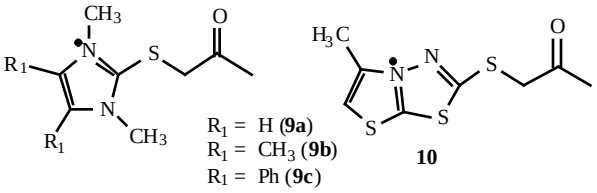


Fig. (6). Structure of 2-[(2-oxopropyl)thio]imidazolium derivatives.

Compound **10** was selected for evaluation in animal models of arterial thrombosis. In the acute canine model of coronary artery thrombosis the infusion of **10** before or shortly after the formation of an occlusive clot resulted in enhanced fibrinolysis in response to t-Pa [128]. The residual thrombus mass was significantly reduced in treated animals compared to the placebo group. However, if the compound was administered 15 minutes after the clot induction in the coronary artery, no benefit of FXIIIa inhibition was observed. Enhanced fibrinolysis, with a short temporal window for FXIIIIa inhibitor administration was also observed in the rabbit model of femoral artery thrombosis [198]. These experiments demonstrate the benefit of combining FXIIIa inhibitors with thrombolytic agents. However, the results emphasize the importance of administering FXIIIa inactivator no later than 8-10 min after the occlusive clot formation in the experimental animals. The role of FXIIIa inhibition in the prevention of secondary occlusion following initial reperfusion has not been specifically investigated in appropriate animal models.

Although developed for FXIIIa, 2-[(2-oxopropyl)thio]imidazolium derivatives can also be used for inactivation of other TGases. Lorand and coworkers [160] demonstrated that 1,3,4,5-tetramethyl-2[(2-oxopropyl) thio]imidazolium chloride (**9b**) and 1,3-dimethyl-4,5-diphenyl-2[(2-oxopropyl)thio] imidazolium trifluoromethyl-sulfonate (**9c**) at 1-2 μM concentration almost completely blocked the transamidating activity of partially purified rabbit lens TGase and at 1 μM concentration prevented the formation of cross-linked β-crystallin dimers.

Dipeptide analogs of 1,2,4-thiadiazoles of general structure shown in (Fig. **7**) were reported to inactivate TG2 isolated from the guinea pig liver [199]. The compounds were designed based on the structure of carbobenzyloxy-L-glutaminylglycine (Cbz-Gln-Gly), a known substrate for this enzyme. An active pharmacophore, 1,2,4-thiadiazole ring that had been previously shown to inactivate cathepsin B [200], was attached to the γ-carboxamide of the glutamine side chain through an amide or an amine linkage. The most potent compound **11** inactivated TG2 with a second order rate constant (k_i/K_i) of approx. 1.5×10^4 $M^{-1}s^{-1}$. Cbz-Glu-Gly substrate afforded partial protection against inactivation, indicating that the inactivator interacts with the glutamine substrate binding site. The detailed mechanism of inhibition was not investigated.

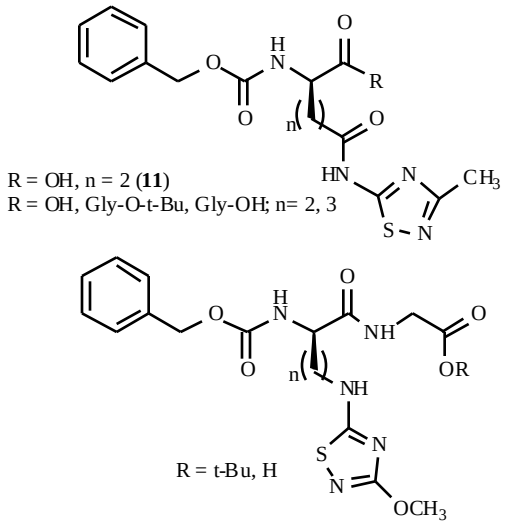


Fig. (7). Structure of FXIIIa inactivators based on 1,2,4-thiadiazole pharmacophore.

Natural Products

Many biologically active compounds have been discovered through screening of natural products. One of the most promising sources of anticoagulants and thrombolytic agents are secretions of animals that use these compounds to their own advantage, such as bloodsucking animals. Scientists at Biopharm Ltd. (U.K.) isolated Tridegin [201], a 66 amino acid (7.3 kDa) polypeptide from the salvary glands of the Giant Amazon Leech, *Haementeria ghilianii*. Tridegin is a very potent inhibitor of FXIIIa and TG2. The K_i for the inhibition of FXIIIa is below that of the enzyme concentration used (9 nM), therefore it could not be accurately determined. The polypeptide also inhibits TG2 but with much lower potency (K_i = 0.23 μM). Tridegin does

not affect other enzymes in the coagulation cascade and exerts its effect by inhibition of FXIIIa only. It is not a substrate for the enzyme and it is not a non-specific thiol blocking reagent. Tridegin does not inhibit other cysteine-dependent enzymes like papain, bromelain or cathepsin C. It was demonstrated that plasma clots prepared in the presence of Tridegin had substantially increased susceptibility to lysis by streptokinase, t-Pa and hementin, a proteolytic enzyme from the same leach [202]. The enhancement of fibrinolysis was even more pronounced when platelet rich plasma was used.

A novel, high molecular weight TGase inhibitor produced by a soil microorganism *Streptomyces lavendulae Y-200* was recently discovered [203]. The inhibitory substance is a glycoprotein of an estimated molecular weight of between 10^4 and 10^5 Da, most likely related to melanin. The inhibitor is competitive with respect to glutamine substrate and has apparent K_i of 1.3 μM for TG2; it also inhibits, with equal potency, FXIIIa, TG1, TG3 and calcium-independent microbial TGase. The observation that its inhibitory activity is stable under acidic, basic and high temperature conditions and is not affected by proteases, suggests that its inhibitory component is not a protein or a sugar. The exact structure and function of this substance remains to be elucidated.

Alutacenoic acids A and B, shown in (Fig. **8**), were isolated as metabolites of fungi *Eupenicillium alutaceum* [204]. Both compounds contain a cyclopropenone ring, a functional group that is rare in a natural product. Naturally occurring alutacenoic acids A and B and a series of their analogs with varied substitution at the carboxy group and the chain length between the ring and the carbonyl group inactivated FXIIIa in a time-dependent manner. The most potent phenethyl amide derivative **12** inactivated FXIIIa with the second order rate constant of 5083 $M^{-1}s^{-1}$. Interestingly, a disubstituted cyclopropenone derivative, had very low inhibitory potency.

A conformational analysis and a docking study of several cyclopropenone derivatives, including the unreactive disubstituted compound, was performed to a model of activated FXIII [205]. As mentioned earlier, a crystal structure of the activated form of the enzyme is not yet available but the structural and biochemical information collected so far provide several clues about the events leading to the activation of the enzyme. Based on this information, an X-ray structure of FXIII zymogen with the activation peptide and the first beta barrel domain removed was used to approximate the activated enzyme and to model several conformations of the inhibitors bound to the active site. Assuming that the cyclopropenone ring is reacting with Cys314 of the enzyme, a mechanism was proposed in which the active site thiolate undergoes a Michael addition to the double bond of the cyclopropenone ring. This is facilitated by the NH group of Trp279, which is proposed to play a role in positioning the cyclopropenone ring and reducing electron density on the carbonyl group. As the reader would recall, the NH group of Trp279 was postulated as a component of the "oxyanion hole" that stabilizes the intermediates formed in the course of transamidation. The proposed inhibition mechanism by derivatives of alutacenoic acids is illustrated in (Fig. **9**).

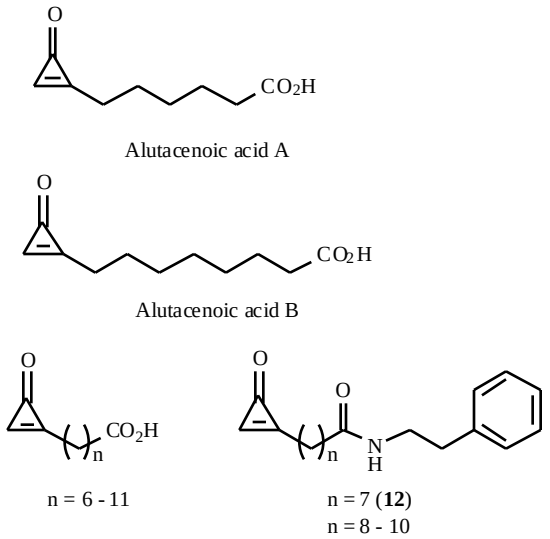


Fig. (8). Structure of alutacenoic acids A and B and their synthetic derivatives.

A low molecular weight, nonpeptide FXIIIa inhibitor, ZG-1400, was isolated from aerobic fermentation of fungal culture *Penicillium roseopurpureum* [206]. The compound was purified, characterized and determined to be an (-)R-enantiomer of cis-resorcylide (**13**). The structure of ZG-1400 is shown in (Fig. **10**). The mechanism of FXIIIa inhibition by ZG-1400 was not studied and only IC_{50} values were reported. The compound inhibited cross-linking of biotynylated fibrin to fibronectin and was approx. 50 times more potent than non-specific thiol reagents iodoacetamide and iodoacetic acid and 5 times more potent than naturally occurring FXIIIa inhibitor, cerulenin schown in (Fig. **10**) [207]. ZG-1400 did not show any selectivity towards FXIIIa over TG2.

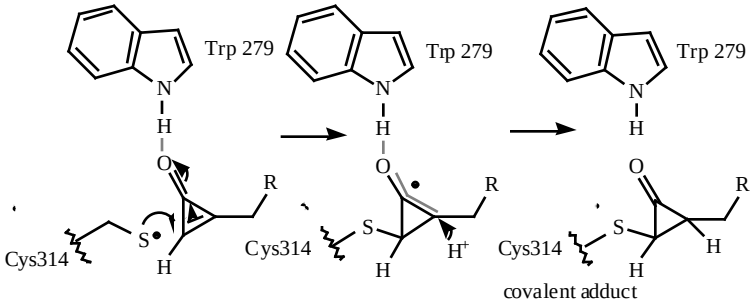
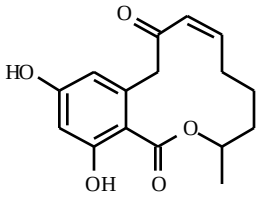


Fig. (9). Proposed mechanism of FXIIIa inhibition by derivatives of alutacenoic acid.

Immunoinhibition of FXIIIa

Monoclonal antbodies (Mab) generated against selected epitopes on a target enzyme are remarkably specific and potent enzyme inactivators. Pursuing this approach, Reed and coworkers [208] developed Mab 9C11, a murine monoclonal antibody against human platelet FXIII. The antibody reacted specifically with both plasma and platelet FXIII but it did not bind well to SDS-denatured FXIII indicating that it recognized a conformationally determined epitope. Fab fragment of Mab 9C11, as well as the whole immunoglobulin, inhibited FXIIIa in a dose-dependent manner. The IC_{50} for the Fab fragment was approximately 5 nM [208]. The antibody inhibited the process of activation of FXIII by thrombin and did not affect the enzyme that has been already activated. Mab 9C11 inhibited fibrin cross-linking in whole plasma clots and the cross-linking of α_2-antiplasmin to fibrin. Plasma clots made *in vitro* in the presence of Mab 9C11 underwent markedly accelerated fibrinolysis in response to urokinase. The Mab 9C11 was subsequently used to examine the contribution of FXIIIa-catalyzed reactions to the fibrinolytic resistance of experimental pulmonary emboli [209]. Whole blood clots were produced exogenously in the presence and in the absence of Mab 9C11 and embolised in the lungs of ferrets. The rates of both endogenous and t-Pa induced fibrinolysis was examined. In addition, a peptide capable of selective inhibition of the cross-linking of α_2-antiplasmin to fibrin was used to differentiate between the effects of fibrin cross-linking and the covalent attachment of α_2-antiplasmin on fibrinolytic resistance of the emboli. It was found that the inhibition of FXIIIa tripled the rate of endogenous thrombolysis in experimental animals. The clots made in the presence of Mab 9C11 lysed more than twice as fast in response to t-Pa treatment. The study demonstrated that inhibition of both FXIIIa catalyzed processes, fibrin cross-linking and the covalent attachment of α_2-antiplasmin to fibrin, greatly facilitates throbolysis.



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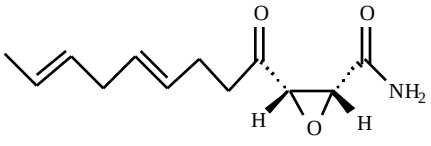

Cerulenin

Fig. (10). Structure of ZG-1400.

6. CONCLUDING REMARKS

While the involvement of TGases in several pathological processes is well documented, the progress in the development of selective inhibitors of these enzymes has been surprisingly slow. Design of low molecular weight inhibitors with a high degree of selectivity is complicated by a fact that TGase substrates are usually large proteins. Therefore, the substrate recognition is most likely not limited to the active site of the enzyme but may also involve structural elements remote from it. TGases have stringent requirements for the glutamine substrate but are not very specific for the amine substrate. For this reason, exploring the features of the glutamine substrate binding site may be a good approach to achieving selective inhibition.

Although significant progress has been made in the elucidation of three-dimensional structures of many TGases, the structure of the active form of FXIII is still unknown. Enzyme structure-based design of FXIIIa inhibitors has to rely on speculations about the protein rearrangement leading to active conformation. Clearly, the X-ray crystal structure of the active form of the enzyme would greatly facilitate these efforts.

Selective inhibitors of TGases will not only assist in the elucidation of various roles of these enzymes in physiological and pathological processes but may also become valuable therapeutics for the treatment of a wide variety of diseases associated with aberrant TGase activity. It can be argued however, that achieving isotype selective inhibition may not be necessary for clinical application. TGase inhibition has been demonstrated to be effective in a guinea pig model of allergic conjunctivitis and murine model of Huntington disease. No harmful effects were reported in those studies. Viable, normal size TG2-/- mice indicate that, under normal conditions, the body has almost bottom level of TG2 activity unless it is triggered by tissue injury or inflammation. Therefore, pan-TGase inhibitors may prove to be effective and safe.

7. ACKNOWLEDGEMENTS

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8. ABBREVIATIONS

AD	=	Alzheimer's disease
$(CAG)_n/Q_n$ -expansion disorders	=	Polyglutamine expansion disorders
CE	=	Cell envelope
CD	=	Celiac disease
DNs	=	Dystrophic neurites
ECM	=	Extracellular matrix
FXIII	=	Factor XIII
HD	=	Huntington's disease
MI	=	Myocardial infarction
Mab	=	Monoclonal antbody
NFT	=	Neuronal neurofibrillary tangles
NIIs	=	Intranuclear inclusions
PHF	=	Paired helical filaments

PLA_2	=	Phospholipase A_2
TGase	=	Transglutaminase
TGF b1	=	Transforming growth factor b1
TG1-TG7	=	Transglutaminases 1 through 7

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