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Semin Immunopathol. Author manuscript; available in PMC 2013 July 16.

Published in final edited form as:
Semin Immunopathol. 2012 July ; 34(4): 513–522. doi:10.1007/s00281-012-0305-0.

Role of Transglutaminase 2 in Celiac Disease Pathogenesis

Cornelius Klöck[1,*], **Thomas R. DiRaimondo**[2,*], and **Chaitan Khosla**[1,2]
[1]Department of Chemistry, Stanford University, Stanford CA 94305
[2]Department of Chemical Engineering, Stanford University, Stanford CA 94305

Introduction to Transglutaminase 2 (TG2) Chemistry and Biology

Biochemical properties

Transglutaminase 2 (TG2) is a 78 kDa, calcium dependent enzyme in mammals. A member of the transglutaminase family, it catalyzes the post-translational modification of selected glutamine residues on the surface of its protein or peptide substrates. The catalytic mechanism of transglutaminase 2 shares some similarities with cysteine proteases (Figure 1), where a thiol residue (Cys277 in human TG2) in the active site attacks the glutamine carboxamide, releasing ammonia. The resulting thioester intermediate can then be attacked by a variety of biological nucleophiles, typically an ε-amino group from a surface lysine residue of a second protein or peptide substrate. The overall reaction results in the formation of a stable ε(γ-glutamyl)-lysine isopeptide bond (a.k.a. "cross-link") that is resistant to proteolysis [1]. Alternatively, the acyl-enzyme intermediate can be hydrolyzed, leading to the net conversion of a glutamine residue into a glutamic acid; this transformation has relevance to celiac disease (*vide infra*).[2] Like most other mammalian transglutaminases, TG2 is a multifunctional protein with other biochemical properties. Perhaps most notably, it binds to guanine nucleotides and functions as a G-protein in signaling processes involving selected 7-transmembrane receptors [3]. It has also been reported to exhibit protein disulfide isomerase and serine/threonine kinase activities.[4, 5]

Localization

TG2 is abundantly expressed in many organs including, for example, the liver, heart, intestine, as well as blood cells such as erythrocytes [6]. It is found in both intracellular and extracellular locations [7]. Inside the cell, TG2 is especially abundant in the cytosol, but can also be found in mitochondria [8, 9] and the nucleus [10, 11]. It should be noted that, notwithstanding its localization in the mitochondria and the extracellular matrix, TG2 does not possess an N-terminal export sequence. Instead, it is thought to be externalized as a complex with β1-integrin via recycling endosomes [12] and/or cell surface shedding of heparan sulfates [13]. In the extracellular matrix, TG2 binds tightly to fibronectin via a 42 kDa gelatin binding domain. Because TG2 contributes to gluten immunotoxicity by modifying selected peptides, the pathogenesis of celiac disease is presumed to depend upon the activity of extracellular TG2 (*vide infra*).

Biological function

A variety of physiological roles have been ascribed to TG2, and have been extensively reviewed elsewhere [7, 6, 14–19]. In the intracellular environment, TG2 is thought to play a pro-apoptotic as well as an anti-apoptotic role, depending on the cellular context and

Address correspondence to: Chaitan Khosla (khosla@stanford.edu).
*These authors contributed equally to this work

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biological cues [20]. Extracellular TG2 is believed to be involved in cell adhesion [21, 22], matrix assembly [18, 23], wound healing [15, 24, 25], receptor signaling [26], and a variety of cellular behaviors including proliferation, invasion, motility and survival [27, 28]. Some of these biological functions have been shown to depend upon the cross-linking activity of the enzyme; others are not. Notwithstanding this diversity of putative biological roles, TG2 knockout mice are developmentally and reproductively normal [29–31]. In this review we will necessarily limit our discussion to only those functions that have compelling relevance to celiac disease pathogenesis.

Post-translational regulation

The expression of TG2 is elaborately regulated in response to a number of transcriptional signals, as reviewed elsewhere [28]. In light of its ubiquity, abundance, and relatively non-specific recognition of glutamine-bearing substrates, the transglutaminase activity of TG2 must also be tightly controlled at the post-translational level. From the perspective of understanding celiac disease pathogenesis, it must be understood that TG2 in the small intestinal mucosa is predominantly inactive under normal physiological conditions [32–35]. It must therefore be activated. A full understanding of the post-translational mechanisms underlying TG2 activation is therefore critical.

The best understood post-translational regulators of cross-linking activity are Ca^{2+} ions, guanine nucleotides (GTP and GDP), and the redox environment of the protein. Transglutaminase activity of full-length TG2 requires Ca^{2+} ion binding. Up to six Ca^{2+} ions can bind to each molecule of TG2 with an apparent overall dissociation constant of 90 μM [36]. In contrast, TG2 activity is inhibited upon binding to one molecule of GTP or GDP, the dissociation constant being 1.6 μM [37]. The GTP-bound form has one residual high-affinity calcium binding site, and requires high (> 1 mM) concentrations of Ca^{2+} to regain activity [38, 39]. Activation is accompanied by a large conformational change from a compact ("closed") to an extended ("open") structure (Figures 2 and 3) [40, 41]. These regulatory features presumably explain why TG2 is ordinarily inactive inside cells, where the free Ca^{2+} concentration is tightly controlled at very low levels (< 1 μM) and GTP is abundant. However, they fail to explain the inactivity of extracellular TG2, which resides in an environment of relatively abundant Ca^{2+} ions and low GTP/GDP concentration [32]. The latter property is accounted for by the reversible formation of a disulfide bond between vicinal Cys370 and Cys371 [33, 34]. Because the redox potential is ordinarily high in the extracellular matrix, extracellular TG2 is predominantly maintained in a disulfide bonded, inactive state. Its enzymatic activity can be transiently activated by the protein cofactor thioredoxin-1 (TRX). Whereas the spectrum of biological cues that trigger redox-mediated activation of extracellular TG2 remains to be understood, interferon-γ has been shown to elicit a burst of TRX release from monocytic cells [34]. Interferon-γ also activates extracellular TG2 associated with enterocytic T84 monolayers via a PI3-kinase dependent mechanism [35]. As discussed below, this observation has implications for celiac disease pathogenesis. The transglutaminase activity of intracellular TG2 can also be induced in response to certain stresses [17], although the mechanisms by which this occurs remain poorly understood. Short splice variants of TG2 with C-terminal modifications have been detected in various cells and tissues [42–45]. As these variants appear to be independent of GTP and have a lower calcium requirement than full-length TG2 [43, 46, 47], they may be the sources of intracellular catalytic activity of TG2.

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Is the Transglutaminase Activity of TG2 Required for Celiac Disease Pathogenesis?

At present, a causative link between the transglutaminase activity of TG2 and celiac disease pathogenesis has not yet been definitively established. Nonetheless, a large body of evidence supports this hypothesis. First, TG2 has remarkably high substrate specificity for immunopathogenic gluten peptides. Second, recognition of these gluten peptides by the disease associated HLA-DQ2 or -DQ8 is strongly enhanced by TG2-catalyzed deamidation. Third, interferon-γ is the predominant cytokine secreted when DQ2-restricted, gluten-responsive T cells derived from the celiac intestine are activated [2]. As discussed above, interferon-γ also triggers TRX-mediated activation of extracellular TG2, thereby plausibly establishing an auto-amplificatory loop for gluten-mediated inflammation. Last but not least, chronic exposure of celiac patients to dietary gluten is invariably accompanied by the production of autoantibodies against TG2. As elaborated below, whereas the mechanistic underpinnings of some of these phenomena are relatively well understood, others are not so. A deeper understanding of these TG2-related events will not only enable definitive verification of the hypothesis that transglutaminase activity is necessary for gluten induced pathogenesis in celiac disease, but could also cast fundamentally new light on the biological function of TG2.

Much is known today about the structural basis for gluten recognition by TG2. Although glutamine residues are abundant in this family of polypeptides, and non-enzymatic (acid-promoted) deamidation occurs non-specifically [48], TG2 catalyzes highly selective deamidation of glutamine residues occurring in specific sequence motifs [49–51]. In particular, the repetitive sequence motif QXPZ, where X is any residue and Z is a hydrophobic residue, is a favored site of TG2-catalyzed deamidation. The X-ray crystal structure of TG2 bound covalently to a pentapeptide mimic of this substrate provides a clear rationale for such preference [40]. It also sets the stage for structure-based design of small molecule TG2 inhibitors that could prove pivotal in pharmacologically testing the causal link between TG2 activity and pathogenesis in human patients.

The discovery of the remarkable concordance between TG2 specificity for gluten peptides and the high affinity of the resulting deamidated peptides for HLA-DQ2 (or – DQ8) inaugurated a fundamentally new chapter in our understanding of celiac disease pathogenesis [52–55]. With the solution of high-resolution X-ray crystal structures of representative deamidated gluten peptides in complex with DQ2 [56] and DQ8 [57] (Figure 4), the pathogenic significance of the distinctive pattern of proline residues in proteolytically resistant gluten peptides has been clarified. Equally important is the TG2-catalyzed introduction of a negative charge at a precise location in a gluten peptide (Table 1). Negatively charged carboxylate residues at P4 or P6 in a DQ2 ligand or P9 in a DQ8 ligand anchor these peptides tightly in the corresponding HLA binding grooves (Table 2). The engineering of a synthetic T cell antigen that is capable of triggering a TG2 dependent autoimmune response via an alternative class II major histocompatibility complex in an experimental animal would represent a powerful, albeit challenging, test of the importance of TG2 activity in celiac disease pathogenesis.

In contrast to the mechanistic clarity summarized above for gluten antigen recognition by TG2 and recognition of deamidated antigens by DQ2 or DQ8, very little is known thus far about the structural basis for recognition of oxidized TG2 by TRX. Because the high-resolution structures of both proteins have been solved, understanding this selective protein-protein interaction at an atomic level is entirely feasible. In turn, such insights could enable the design of chemical tools to modulate the efficiency extracellular TG2 activation, thereby

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attenuating gluten mediated T cell inflammation in the small intestines of celiac patients as well as animal models of the disease.

Perhaps most intriguingly, the role of serum IgA and IgG antibodies to TG2 represents a major frontier for molecular investigations into celiac disease pathogenesis. Such autoantibodies are hallmarks of CD, and are in fact used as diagnostic markers [58, 59]. However, our understanding of the role of these autoantibodies in the onset and progression of disease pathogenesis is very limited. Anti-TG2 antibodies are prolifically secreted in the small intestinal mucosa of celiac patients in response to dietary gluten [60]. They are preferentially localized in the sub-epithelial layer, where they adhere tightly to extracellular TG2 on fibroblasts and on the basement membrane of the small intestine [61–63]. Patient derived anti-TG2 autoantibodies induce enterocyte proliferation [64, 65], inhibit enterocyte differentiation [64], and modulate epithelial barrier function [60, 66, 67]. Thus, it is entirely possible that anti-TG2 antibodies promote intestinal crypt hyperplasia and villous blunting, two hallmarks of a celiac lesion. Proof of this hypothesis awaits elucidation of the docking mode(s) of disease-specific autoantibodies on TG2 and the biochemical implications of these antigen-antibody interactions. The recent discovery of a celiac disease-specific conformational epitope on the surface of TG2 may provide an important starting point for such investigations [68].

Physiological and Toxicological Consequences of TG2 Activation

As summarized above, arguably the most important toxicological consequence of extracellular TG2 activity in the celiac small intestine is the activation of gluten-dependent inflammatory T cells. However, it has also been reported that a certain gluten peptide (designated p31–43) activates intracellular TG2, leading to the degradation of the anti-inflammatory peroxisome proliferator-activated receptor (PPARγ)[69, 70]. PPARγ is a hormone receptor produced by several cell types, including epithelial cells, which negatively regulates inflammatory responses [71] and modulates oxidative stress [72, 73]. Therefore, activation of intracellular TG2 in the celiac small intestine can also be expected to contribute towards gut inflammation. However, because this phenomenon neither involves DQ2/8 nor does it involve disease-specific T cells, it remains to be explained if and how gluten mediated activation of intracellular TG2 by this mechanism is specific to the celiac small intestine.

More generally, activation of intracellular and/or extracellular TG2 can be expected to have multifactorial effects on mucosal biology. The connection between these biological consequences of TG2 activation and the wide range of intestinal and extra-intestinal symptoms observed in celiac patients represents an important direction for future research. Furthermore, if TG2 inhibition does prove to be an effective way to control the disease, then it will also be important to understand the physiological and toxicological consequences of inhibiting this ubiquitous but mostly inactive enzyme.

Models for Investigating the Causes and Consequences of TG2 Activation

It should be noted that, until now, the causes and consequences of gluten-dependent TG2 activation could only have been investigated in the celiac patient, a daunting option. Recently however, encouraging steps have been taken towards the development of engineered mice in which dietary gluten consumption, DQ8-restricted T cell response, intestinal villous atrophy, and autoantibody formation are phenotypically linked.

Intestinal epithelial cell culture models have also been used to investigate fundamental aspects of TG2 activity as it relates to celiac disease. For example, exposure to IFN-γ increases both gluten peptide permeability [74] and the transglutaminase activity of

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extracellular TG2 [35] in a simple T84 enterocytic cell culture model. More advanced studies along these lines could benefit enormously from the application of emerging methods to culture primary intestinal epithelial cells [75, 76] and especially methods to co-culture enterocytes with fibroblasts and cells derived from the immune system.

An essential aspect of modeling the role of TG2 in celiac disease is the requirement for HLA-DQ2 or -DQ8. Towards this end, transgenic mice have been generated that express both these human class II MHC receptors [77, 78]. Already, these humanized mice are providing critical insights into the role of TG2 in celiac disease pathogenesis. For example, in HLA-DQ8 mice, whereas TG2-catalyzed deamidation facilitates the recruitment of a broad repertoire of inflammatory T cells in response to gluten antigens, a limited range of T cells is also provoked in response to native gluten peptides both in mice and humans [79]. Nonetheless, even though these humanized mice react strongly to an intravenous gluten antigen challenge, the hallmark of celiac disease - villous atrophy - is not observed [77, 78, 80]. Moreover, oral gluten is not an effective antigen in either mouse strain. In contrast, transgenic mice that constitutively over-express IL-15 in the gut show many of the features associated with the human condition, including gluten-dependent defects in the regulation of intraepithelial lymphocytes, spontaneous inflammation of the small intestine, villous atrophy and crypt hyperplasia [81–83]. While these animals are the most comprehensive models for celiac disease thus far, the status of TG2 activity in their small intestinal mucosa remains to be elucidated. If it can be shown that mucosal TG2 activity is upregulated constitutively or in response to gluten in these animals, then many of the above questions could be powerfully addressed using this model.

TG2 as a drug target for celiac disease

With its ubiquitous nature and its diverse functions, there might be concern that pharmacological inhibition of TG2 would give rise to undesired target-related effects. While the physiological consequences of TG2 inhibition have not yet been studies in detail, the absence of a strong phenotype in TG2 knockout mice suggests that TG2 inhibition should be tolerated [29–31]. In addition, in the context of CD, the location of the pathological enzymatic activity in the lamina propria would present an opportunity for engineering TG2 inhibitors for a localized action to mediate any undesired consequences of systemic pharmacological inhibition [84].

The prospect of TG2 as a potential target for celiac disease has motivated the development of several classes of TG2 inhibitors as tools and potential lead compounds (Figure 5). Three general inhibition strategies have emerged for blocking the catalytic activity of TG2, targeting the active site from either the acyl donor or the acyl acceptor substrate binding pockets or targeting an allosteric site [85].

Acyl-donor pocket active site inhibitors

The most widely utilized approach to inhibiting TG2 activity relies on blocking the active site from the acyl donor substrate binding pocket. These inhibitors are substrate competitive; both substrate-mimetic peptidic scaffolds and small molecules have been developed [85].

The peptidic scaffolds that have been developed usually bear a glutamine-isostere with a reactive functionality, such as epoxy or diazo-ketones or Michael acceptor systems (**A; B**) that covalently modify the active site Cys277 [40, 86–88]. A class of irreversible inhibitors around the mildly electrophilic 3-bromo-4,5-dihydroisoxazole (DHI) warhead motif on a single amino acid scaffold has been extensively developed (**C**)[89–91]. Small molecule inhibitors binding to the active site include the cinnamoyl ketones (**D**)[92, 93], the

acrylamido-arylsulfonamides (**E**)[94] the irreversible thioimidazolium derivatives (**F**) [88, 95] and the aminoethyl-arylketones (**G;H**) [96, 97]

Acyl-acceptor pocket active site inhibitors

A number of pseudo-substrate inhibitors that compete with acyl-acceptor substrates have been disclosed, mostly aliphatic amines mimicking lysine.[85]. However, it is unclear whether the acyl-acceptor binding pocket of human TG2 represents an adequate hot-spot for targeting small molecule inhibitors.

Allosteric inhibitors

Although TG2 is physiologically regulated by allosteric binding of GTP, this approach has not been extensively used in the design of TG2 inhibitors. It appears that, unlike in the case of kinases, where the nucleotide binding pocket is the site most site frequently targeted by inhibitors mimicking the nucleobase interactions, the respective pocket in TG2 only tolerates close GTP analogues [98]. Two classes of TG2 inhibitors have been reported to target an allosteric site on TG2, the thienopyrimidinone hydrazides (**I**) [99] and the acylidene oxindoles (**J**)[100]. The former class appears to bind to either the GTP site or a site that is coupled to it. However, for neither of these two inhibitor classes has the binding site been definitively identified [101].

Notwithstanding the plethora of potential TG2 inhibitors, only few have been validated in living cell or animal systems. Inhibitors based on the DHI warhead (**C**) have been used in cell culture to probe and inhibit TG2 activity, as well as in mice to block poly(I:C) mediated TG2 activation [32, 90, 91, 102]; in neither case were pronounced toxic effects observed, even in response to multiple dosing regimens. The thioimidazolium-derivative (**F**) has also been used in experiments with celiac patients' biopsies [103]. Further studies are warranted to understand target-associated and compound-specific toxicological effects.

Conclusion

A number of lines of evidence suggest that TG2 may be one of the earliest disease-relevant proteins to encounter immunotoxic gluten in the celiac gut. These and other investigations also suggest that the reaction catalyzed by TG2 on dietary gluten peptides is essential for the pathogenesis of celiac disease. If so, several questions are of critical significance. How is TG2 activated in the celiac gut? What are the disease-specific and general consequences of activating TG2? Can local inhibition of TG2 in the celiac intestine suppress gluten induced pathogenesis in a dose-responsive manner? And what are the long-term consequences of suppressing TG2 activity in the small intestinal mucosa? Answers to these questions will depend upon the development of judicious models and chemical tools. They also have the potential of yielding powerful next-generation drug candidates for treating this widespread but overlooked chronic disease.

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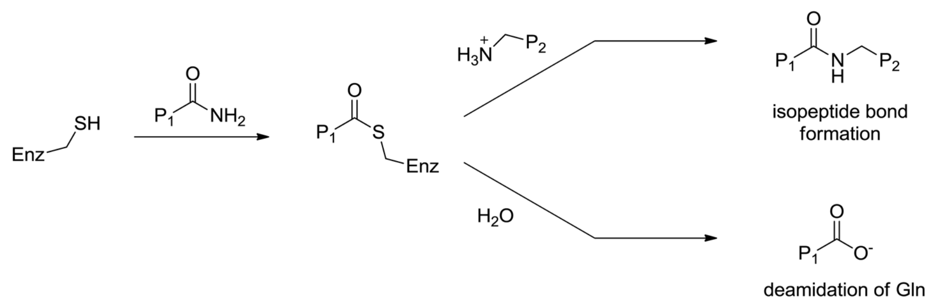

Figure 1.
In the catalytic mechanism of transglutaminases, the active site cysteine (Enz-SH) attacks the glutamine of the acyl donor substrate P_1, forming an intermediate thioester that can either be attacked by the amine of an acyl acceptor substrate P_2, yielding a peptide bond or be hydrolyzed, corresponding to the net deamidation of P_1.[107] The acyl donor substrate P_1 bearing a glutamine is typically a protein but small peptides are recognized as well. The acyl acceptor substrate P_2 is typically a lysine sidechain on a protein or small peptide but can be replaced by small molecule aliphatic amines. [108–110]

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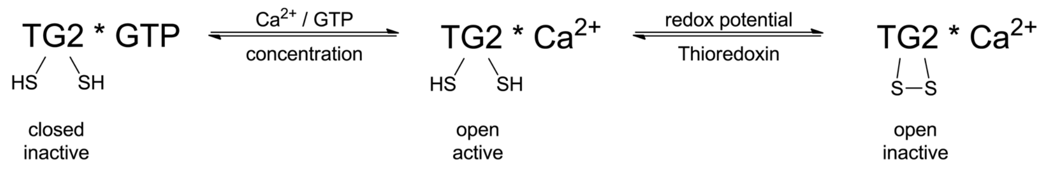

Figure 2.
States of TG2 under different physiological conditions and their activity.

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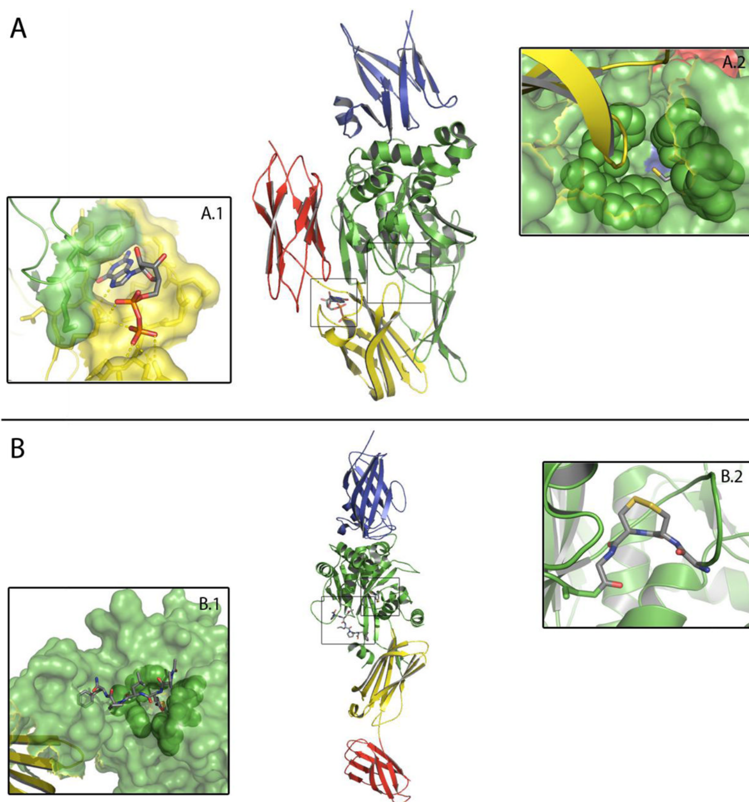

Figure 3.

Crystal structures of TG2 in closed (**A**, PDB 1K3V)[41] and open (**B**, PDB 2Q3Z)[40] conformations. Domains are colored as follows: N-terminal domain (blue), catalytic domain (green), first C-terminal β-barrel (yellow), second C-terminal β-barrel (red). A: In the closed conformation, GDP is bound to the nucleotide binding site (insert A.1) at the interface between the catalytic domain and the first C-terminal β-barrel. TG2 is inactive, because a loop from the first C-terminal β-barrel (yellow) protrudes towards the acyl donor site tunnel. The tunnel is lined by Trp241, Gln276, Trp278, Trp332 and Phe334 (green spheres), and harbors the active site Cys277 at its bottom (insert A.2). B: In the open conformation, the loop from the first C-terminal β-barrel has moved to allow substrate access to the active site (insert B.1), which is occupied by an inhibitor irreversibly bound to Cys277. The vicinal disulfide bond between Cys370 and Cys371 is depicted in insert B.2.

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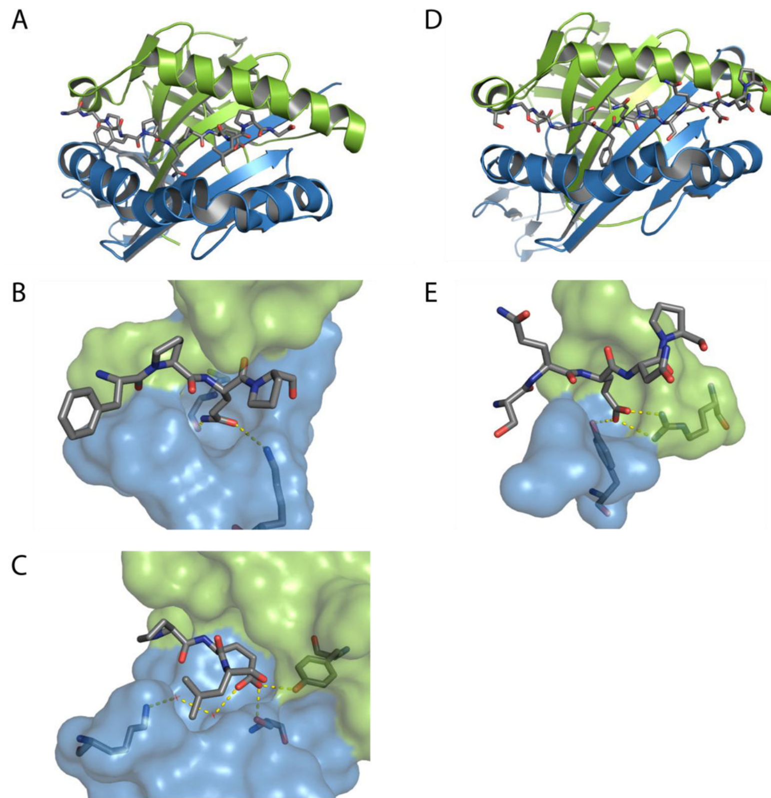

Figure 4.

A: Crystal structure of HLA-DQ2 (A; PDB 1S9V; α-chain in green, β-chain in blue) in complex with the αI-gliadin peptide (stick representation). The P4 and the P6 sites preferentially bind deamidated gliadin peptides. B: The P4 glutamine of αI-gliadin is shown interacting with Lysβ71. Deamidation of this residue by TG2 would stabilize this interaction. C: The deamidated P6 glutamate of αI-gliadin is shown hydrogen bonding with Tyrβ9 and Serβ30 and interacting with Lysβ71 through two water molecules.[56] D and E: Crystal structure of HLA-DQ8 (PDB 2NNA; α-chain in green, β-chain in blue) in complex with α2-gliadin:223–240. The P9 site of HLA-DQ8 binds a deamidated glutamine residue through two salt bridges with Argα76 and a hydrogen bond with Tyrβ37.[57] See also Table 1.

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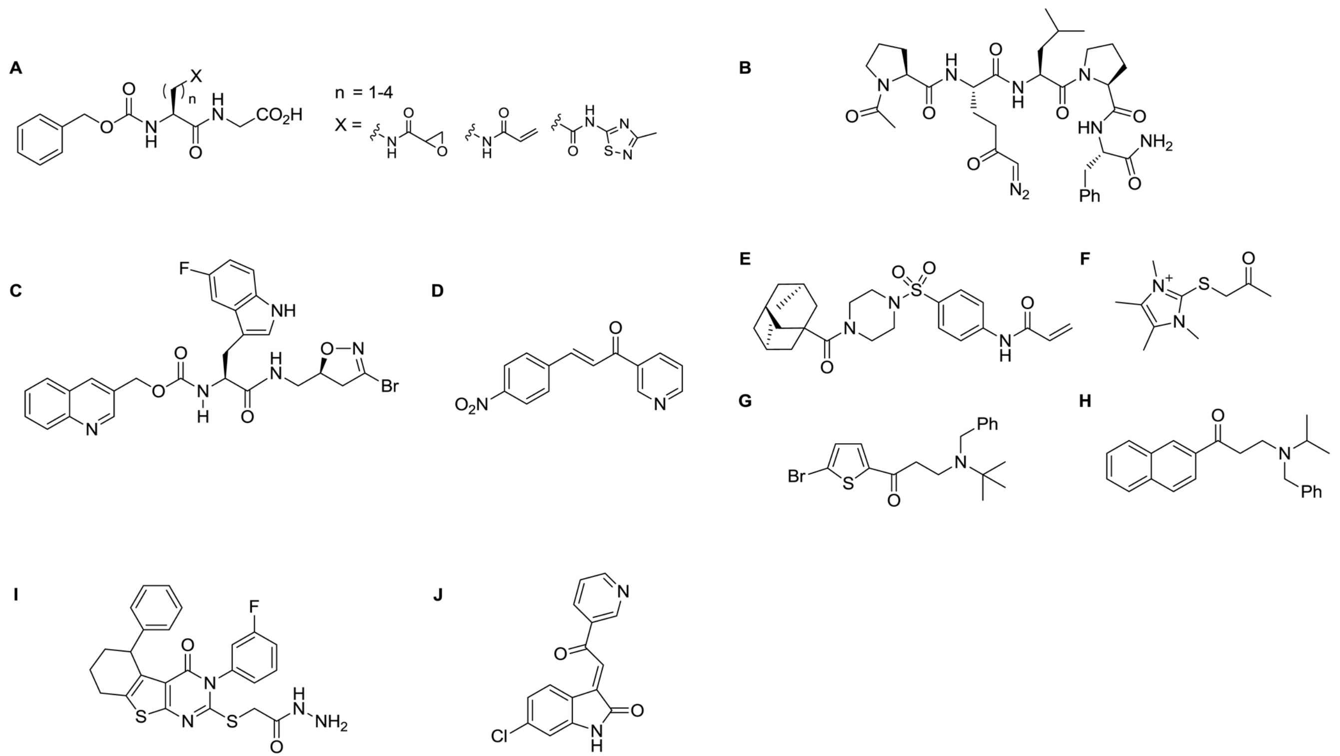

Figure 5.
Structures of selected TG2 inhibitors

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Table 1

Amino acid sequences of immunotoxic gluten peptides and their specificity for TG2 mediated deamidation [51, 104]

Amino Acid Sequence Immunotoxic Gluten Peptides	TG2 Specificity k_{cat}/K_M ($min^{-1}mM^{-1}$)
LQLQPF(PQPQLPY)$_3$PQPQPF	440
QLQPFPQPQLPYPQPQS	260
PQPQLPYPQPQLPY	300
QLQPFPQPQLPY	66
PQQPQQSFPQQQRP	61
γ-Fibrinogen Peptide	
TIGEGQQHHLG	63

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Table 2

Immunotoxic deamidated gluten epitopes and their EC_{50} for binding to the HLA-DQ2 and –DQ8 antigen receptors [57, 105, 106]

Gluten Peptide Gluten Epitope	Immunotoxic Core Sequence	HLA-DQ2 Binding EC_{50} (μM)	HLA-DQ8 Binding EC_{50} (μM)
Gliadin-α2	PQPELPYPQ	15	-
Gliadin-α9	PFPQPELPY	8	-
Gliadin-α20	FRPEQPYPQ	30	-
Glu-5	EXPEQPQQF	100	-
Gliadin-γ2	PYPEQPEQP	65	-
Gliadin-γ1	PQQSFPEQE	14	-
Gliadin-γ30	IIQPEQPAQ	10	-
Glt-17	PFSEQEQPV	25	-
Gliadin-α1	EGSFQPSQE	-	~2