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Tissue transglutaminase: An emerging target for therapy and imaging

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Dedicated to Professor Jörg Steinbach, Dresden-Rossendorf, on the occasion of his 60th birthday

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ABSTRACT

Tissue transglutaminase (transglutaminase 2) is a multifunctional enzyme with many interesting properties resulting in versatile roles in both physiology and pathophysiology. Herein, the particular involvement of the enzyme in human diseases will be outlined with special emphasis on its role in cancer and in tissue interactions with biomaterials. Despite recent progress in unraveling the different cellular functions of transglutaminase 2, several questions remain. Transglutaminase 2 features in both confirmed and some still ambiguous roles within pathological conditions, raising interest in developing inhibitors and imaging probes which target this enzyme. One important prerequisite for identifying and characterizing such molecular tools are reliable assay methods to measure the enzymatic activity. This digest Letter will provide clarification about the various assay methods described to date, accompanied by a discussion of recent progress in the development of inhibitors and imaging probes targeting transglutaminase 2.

TGase 2—Structure, basic functions and regulation: Tissue transglutaminase (TGase 2) belongs to a family of enzymes (EC 2.3.2.13) that catalyze the Ca^{2+}- and thiol-dependent posttranslational modification of proteins by the generation of isopeptide bonds.[1] These bonds are formed by an acyltransfer (transamidation) reaction wherein a γ-carboxamide group of a protein-bound glutamine residue acts as an acyl donor and a wide spectrum of primary amines act as acyl acceptors, especially protein-bound lysine residues and low-molecular weight polyamines such as putrescine, spermidine and spermine.[1] In addition to transamidation, TGases can also catalyze esterification and hydrolysis reactions of glutamine residues.[2] Beside TGase 2, transglutaminase activity has been observed for several other proteins, that is, factor XIIIa (FXIIIa) of the blood clotting cascade and TGases 1, and 3–7.[3]

Within the transglutaminase family, TGase 2 is the most frequently occurring member in eukaryotes and is present in almost all mammalian cells. It is a unique enzyme whose cellular localization, conformations, regulating factors/mechanisms and biochemical functions are in a complex interplay (Fig. 1). In addition to its role as Ca^{2+}-dependent catalyst of the aforementioned transamidation reactions, TGase 2 is able to bind and hydrolyze GTP (and also ATP). These diverse catalytic activities are structurally reflected in a multidomain protein composed of four distinct domains (Fig. 2). An N-terminal β-sandwich domain is followed by a central α/β-domain harboring the acyltransferase activity with Cys277, His335, Asp358 and Trp241 as active site residues. This domain is connected to two consecutive β-barrels located towards the C-terminus. The two β-barrels, together with parts of the α/β transamidase core domain, account for the GTPase activity of TGase 2. The N-terminal β-sandwich does not contribute to catalysis but confers affinity to fibronectin.[4] Five calcium binding sites have been identified in the α/β-domain, which cooperatively bind up to six Ca^{2+} ions per molecule of protein.[5] Through its GTPase site, TGase 2 can act as a G protein (designated G_h) mediating the signal transduction of α_1-adrenergic- (α_{1B} and α_{1D}), oxytocin- and thromboxane A_2-receptors to the primary effector phospholipase Cδ.[6] In this context, GTP/GDP and Ca^{2+} ions act as inverse regulators of the GTPase and transamidase activities of TGase 2. X-ray crystallographic investigations have shown that TGase 2 adopts a closed, transamidase-inactive conformation when GDP is bound, in which the β-barrel domains interact noncovalently with the α/β core domain (Fig. 2, left).[7] Moreover, an X-ray

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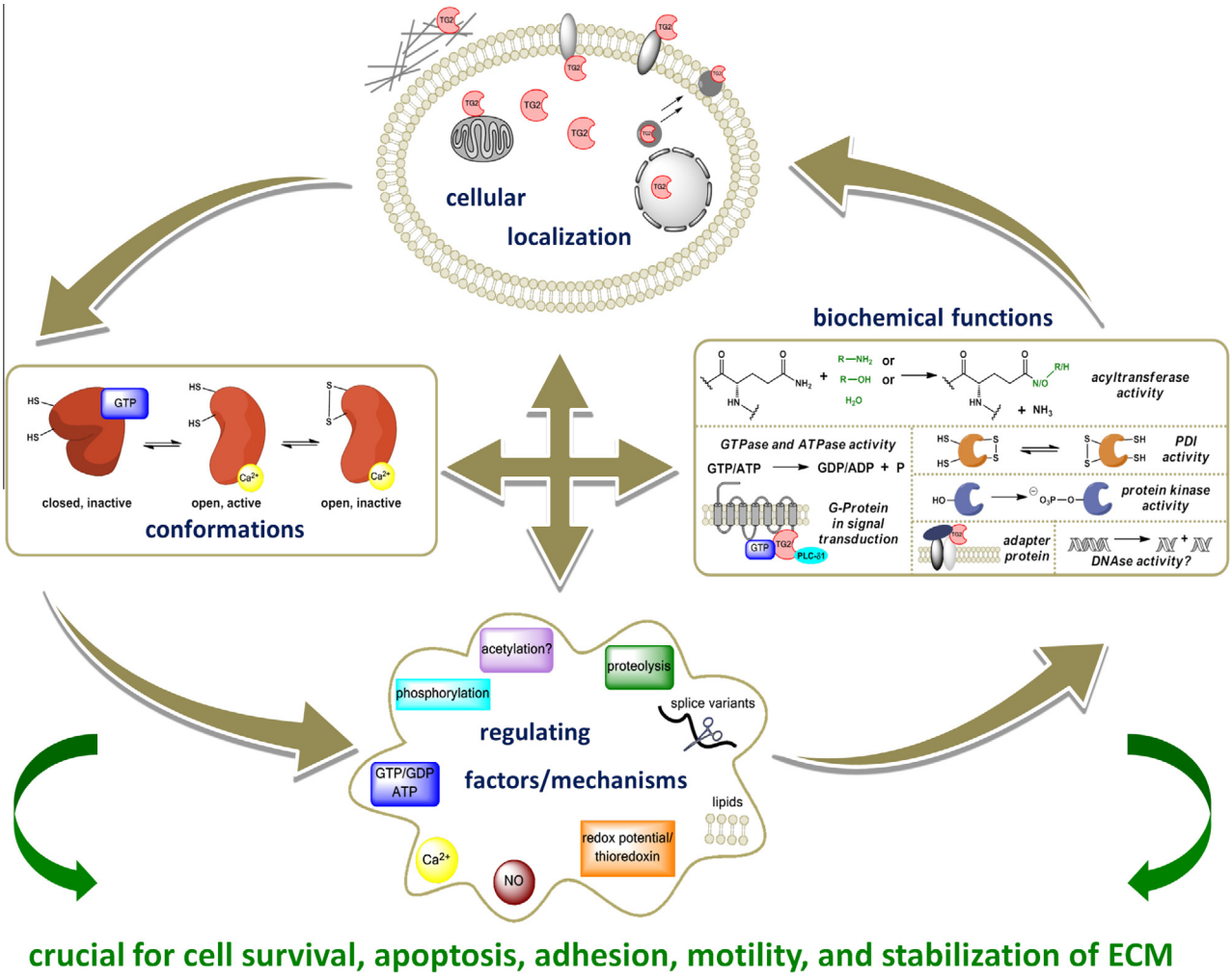


Figure 1. Representation of the complex interplay in TGase 2 physiology. TGase 2 in perinuclear recycling endosomes is depicted as . See text for further details.

structure in the presence of Ca^{2+} ions has been solved for a TGase 2 in complex with an irreversible inhibitor targeting the transamidase site.[8] Within this complex, TGase 2 adopts an open conformation in which the transamidase domain is accessible for substrates (Fig. 2, right). This conformational switch was confirmed by electrophoretic investigations via native polyacrylamide gel electrophoresis (nPAGE) and kinetic capillary electrophoresis. In these experiments two different TGase 2 forms were observed, whose concentrations depend on the presence of Ca^{2+} ions, GDP and irreversible inhibitors.[8,9] As well as the aforementioned transamidase and GTPase activities, TGase 2 exhibits several additional biochemical functions and has thus to be considered as a multifunctional protein. As such, it can also act as a protein disulfide isomerase (PDI, Ca^{2+}- and GTP-independent),[10] in which role it is probably responsible for the correct folding of proteins constituting the mitochondrial respiratory chain.[11] TGase 2 can also act as a protein kinase with insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) and retinoblastoma protein (Rb) as confirmed substrates.[12] While these enzymatic functions are widely accepted, the DNA nuclease activity of TGase 2 suggested by Takeuchi et al. has yet to be confirmed.[13]

The diverse catalytic activities are strictly regulated by different mechanisms. As mentioned above, the transamidase activity of TGase 2 is activated by Ca^{2+} ions and inhibited by GTP. A further low-molecular weight factor that influences TGase 2 has been identified as nitric oxide, which can abolish the transamidase activity by Ca^{2+}-dependent S-nitrosylation of multiple cysteine residues.[14] Additionally, TGase 2 can be influenced by posttranslational modifications, among which regulation by disulfide formation is probably best understood.[15] Disulfide bond formation in TGase 2 does not involve active site Cys277 but three different cysteine residues, that is, cysteines 230, 370 and 371. Cys230 has been shown to form an initial disulfide bond with Cys370 which undergoes thiol-disulfide exchange with Cys371, resulting in a more stable vicinal disulfide.[16] The oxidized, disulfide-bonded form of the enzyme is acyltransferase-inactive and can be activated by thioredoxin.[17] It has been found that TGase 2 can undergo phosphorylation at Ser216 (probably mediated by protein kinase A),[18] which inhibits the transamidase activity and mobilizes its protein kinase activity.[12b] Potentially, TGase 2 can be regulated by N-acetylation of lysine side chains, as the incubation with mild acetylating agents attenuates its transamidase activity.[19] Furthermore, regulation can occur by limited proteolysis[20] and interaction with phospholipids.[21] Beside these regulatory mechanisms, all of which act on the protein level, TGase 2 can also be subject to transcriptional regulation. To this effect, TGase 2 does not only occur as full-length enzyme but also in C-terminally truncated variants resulting from alternative splicing of its primary transcript consisting of 13 exons. These splice variants are referred to as TGase 2_{v1}, TGase 2_{v2}, tTGH (also known as TGase-S) and tTGH2 with a length of 674, 645, 548, and 349 amino acids, respectively, compared to 687 amino acids for the full-length TGase 2. The truncation of the first three splice variants affects only the GTPase domain, resulting in altered

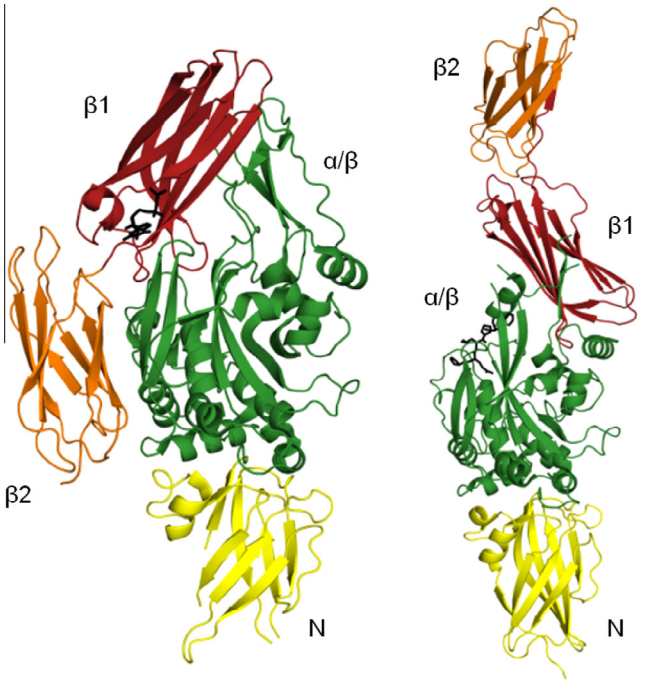


Figure 2. X-ray crystal structures of the closed (left) and open (right) conformations of human TGase 2 (hTGase 2) in complex with GDP and the peptidic inhibitor Ac-P(DON)LPF–NH_2 (DON: 6-diazo-5-oxo-L-norleucine), respectively. Fig. 2 was prepared with PyMOL (DeLano, W. L. *The PyMOL Molecular Graphics System*. Version 1.5.0.3 Schrödinger, LLC) using the PDB files 1KV3[7] and 2Q3Z[8], respectively.

properties for GTP binding and hydrolysis which have been reviewed in detail by Lai and Greenberg.[22] In contrast, tTGH2 lacks the GTPase domain completely and exhibits a C-terminally truncated transamidase domain.[22]

Under physiological conditions, intracellular TGase 2 exists predominantly in its transamidase-inactive conformation, due to the low intracellular Ca^{2+} concentration and the high energy load of the cells.[23] However, during situations of unbalanced Ca^{2+} homeostasis, such as apoptosis or wound healing, TGase 2 undergoes rapid activation upon binding of Ca^{2+} ions.[23,24] This initiates transamidation reactions in dying cells leading to the extensive cross-linking of intracellular proteins, which stabilizes the dying cell and thus prevents the release of harmful and immunogenic degradation products.[25] Although TGase 2 is predominantly a cytosolic protein, it also occurs in the nucleus, mitochondria as well as in the extracellular matrix (ECM) and can be associated with proteins of the cellular membrane (both inside and outside).[26] Release of TGase 2 into the extracellular matrix probably occurs by a non-classical protein secretion pathway using perinuclear recycling endosomes.[27] In the extracellular environment, TGase 2 is mainly transamidase-inactive despite the presence of Ca^{2+} and low levels of GTP/GDP there.[24,28] One reason for this—amongst others—is probably the modification of certain cysteine residues by disulfide formation or nitrosylation reactions as mentioned before.[14,16] Under the influence of inflammatory stimuli, activation of extracellular TGase 2 can occur by thioredoxin-catalyzed reduction of the disulfide bond between Cys370 and Cys371.[17] Within the extracellular matrix, the enzyme acts either as an adapter protein through association with, for example, fibronectin, integrins, and heparan sulfates affecting cell adhesion, motility and cell survival or as a transamidating enzyme stabilizing the extracellular matrix against mechanic and proteolytic degradation.[4a,28,29]

TGase 2—Implications in human diseases: In the course of elucidating the physiological roles of TGase 2, the involvement of this enzyme in the pathogenesis of a number of diseases was revealed. These include the autoimmune disorder celiac disease,[30] neurodegenerative disorders, especially Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD),[31] various kinds of cancer[32] and diseases related to tissue fibrosis.[33]

Probably the best molecular description for the involvement of TGase 2 in pathological disorders is established for celiac disease, an autoimmune disease that is characterized by an impairment of the small intestine.[34] In the pathogenesis of this disorder, TGase 2 exerts two distinct functions. In the first place, the enzyme deamidates specific glutamine residues in the prolamin fraction of gluten proteins, which results in increased binding affinities of these proteins to the human leukocyte antigen (HLA) serotypes DQ2 and DQ8.[35] Secondly, TGase 2 forms covalent complexes with gluten, leading to activated T cells and finally to an immune response against itself by generation of TGase 2 specific autoantibodies.[35b,36]

The hallmarks of several neurodegenerative disorders are extensive neuronal loss and progressive formation of insoluble protein aggregates in the affected cerebral regions, called amyloid plaques and Lewy bodies in the case of AD and PD, respectively.[37] It has been proven that both TGase 2 expression and its transamidase activity are increased in the brains of persons suffering from these neurodegenerative diseases.[38 and citations therein] Furthermore, the corresponding disease-related proteins such as huntingtin, amyloid Aβ, tau and α-synuclein are good substrates of TGase 2 in vitro.[39] This indicates that TGase 2 may be significantly involved in the pathogenesis of neurodegenerative disorders, with recent results suggesting that TGase 2 catalyzes the formation of soluble neurotoxic protein aggregates rather than insoluble aggregates.[38,40]

Another pathological process related to protein aggregation is cataractogenesis in the eye. There is accumulating evidence that TGase 2 contributes to this process by cross-linking crystallins, that is, the proteins constituting the eye lens, which consequently leads to lens opacification.[41] Interestingly, this seems to correlate with the formation of bis(γ-glutamyl)spermidine cross-links between the crystallin proteins. Increased spermidine concentrations were found to prevent opacification due to favoring the formation of soluble protein-bound mono(γ-glutamyl)spermidines.[41c]

Several studies have demonstrated that TGase 2 is overexpressed in different kinds of cancer,[42] including pancreatic carcinoma,[43] breast carcinoma[44] and malignant melanoma.[45] In this context, elevated levels of TGase 2 in cancer cells have been associated with increased drug resistance and metastasis.[43–46] There is evidence that TGase 2 mediates these properties by influencing the focal adhesion kinase (FAK)/phosphoinositid-3-kinase/AKT-signaling pathway activating FAK and inhibiting the phosphatase and tensin homolog (PTEN), among other pathways.[43,47] Furthermore, TGase 2 causes activation of the nuclear factor κB (NF-κB) by a variety of mechanisms.[48] The most well confirmed one of these mechanisms seems to be the TGase 2-catalyzed polymerization of the inhibitor of NF-κB α protein (IκBα) by intermolecular glutamine-lysine cross-links.[48a,b,f] In addition to this, alternative mechanisms for NF-κB activation that do not require the transamidase acitvity of TGase 2 have been discussed.[48c,e,f] On the other hand, TGase 2 itself is subject to transcriptional control by NF-κB, which leads to a self-stimulating signaling loop.[48f] The close relationship between NF-κB and TGase 2 makes this enzyme a potential target for inhibition. Such inhibition could potentially reverse chemo- and radioresistance, as NF-κB is induced by genotoxic stress elicited by alkylating and intercalating cytostatic agents, DNA antimetabolites, reactive oxygen species and ionizing radiation.[43,49] A further signaling pathway, involving TGase 2 leading to enhanced cell growth has been revealed by Li et al., who demonstrated that activation of the epidermal growth factor receptor (EGFR)

increases the expression and activity of the enzyme. This results in the formation of a ternary complex between TGase 2, keratin-19 and the protein tyrosine kinase Src. The ternary complex, where keratin-19 is presumably connected to TGase 2 as thioester with Cys277, renders Src active conferring increased oncogenic potential to SKBR3 tumor cells.[50] Furthermore, signaling between TGase 2 and EGFR seems to also work in the reverse direction, as it has been recently shown that the enzyme can enlarge the lifespan of EGFR in glioblastoma cells by preventing the ubiquitylation of the intracellular receptor domains through complex formation with c-Cbl, that is, an E3 ubiquitin ligase. In this process, the ability of complex formation with c-Cbl has been attributed to the GTP-bound, closed form of TGase 2.[51]

Not only intracellular signaling pathways, but also communication with other neoplastic and tumor-associated cells and influences from the microenvironment shape tumor growth. TGase 2 has been shown to participate in these processes. In this context, Wang and Griffin recently found a correlation between TGase 2 and S100A4, another Ca^{2+} binding intra- and extracellular protein which is also highly expressed in metastatic tumor cells and linked with tumor progression.[52] Their detailed investigations revealed that S100A4 is a substrate for TGase 2-catalyzed transamidation and that the cross-linking of S100A4 is involved in the activation of the syndecan-4 and α5β1 integrin co-signaling pathway mediated by protein kinase Cα (PKCα), which results in increased cell migration. In addition to discrete molecules, tumor cells can also release specialized extracellular organelles called microvesicles, also known as exosomes or oncosomes, in order to promote tumor development. Their cargo contains proteins and RNA which, when taken up by neighbouring normal cells, can alter the phenotype of these cells leading to enhanced cell survival and aberrant cell growth.[53] It has been demonstrated that TGase 2 plays an important role in the transformation of fibroblasts by microvesicles derived from MDA-MB-231 and U87 tumor cells due to catalyzing the formation of covalently linked fibronectin dimers.[54] Overall, the TGase 2 induced changes can lead, via different signaling pathways, to epithelial–mesenchymal transition (EMT), where immotile epithelial cells are transformed into motile mesenchymal cells.[46b,49e,55] Interestingly, a recent study showed that elevated TGase 2 levels in A5489 lung cancer cells induce the expression of N-cadherin, that is, a mesenchymal marker, to promote EMT.[55c]

Despite the widely described positive correlation of TGase 2 overexpression with tumor progression, some evidence has also indicated that TGase 2 can have an inhibitory effect in certain cancers. It has been demonstrated that TGase 2 expression and transamidase activity is reduced in primary tumors and the surrounding stroma, allowing them to grow and to spread to distant sites.[56 and citations therein] This is presumably linked to the functions of TGase 2 with regards to cell adhesion, motility and ECM stability. It has been shown that TGase 2 in the extracellular space can activate the G-protein coupled orphan receptor GPR56 via its C-terminal β-barrel domains, which led to attenuation of metastasis in melanoma[57] even though TGase 2 is not the only ligand for this receptor.[58] This may explain, in part, the antitumor effects of the enzyme. Moreover, Jones et al. observed decreased tumor growth and sometimes even tumor regression upon injection of TGase 2 into CT26 colon carcinoma-bearing mice.[59] A recent study confirmed that reduced TGase 2 expression in CT26 colon carcinoma occurs during tumor growth and progression.[60] In addition to its extracellular effects, a possible intracellular role of the transamidase activity of TGase 2 in regulating the metastatic potential has been demonstrated where protein-linked γ-glutamyl-polyamine modifications in B16 melanoma cells were compared in cells which had either high or low metastatic potential.[61] The findings of this study led the authors to hypothesize that a low transamidase activity and a concomitantly high content of intra- and extracellular polyamines results in the preferential formation of mono(γ-glutamyl) derivatives of polyamines, which correlates with a highly metastatic phenotype. This directly maintains cellular plasticity and finally favors cellular motility linked to metastasis.[61,62] In this regard, it is worth mentioning that it was recently established that TGase 2 can mediate aminoalkylation of distinct Gln residues in axonal tubulin, resulting in stabilization of microtubules, again suggesting a link between the enzyme and cell motility.[63]

In summary, there is emerging evidence that TGase 2 is implicated in tumor progression. However, the particular roles of TGase 2 and the importance of its transamidase activity in carcinoma seems to depend on the tumor stage and cancer type, probably resulting from the plethora of binding partners and substrates for this enzyme, together with its different intra- and extracelluar localizations and splice variants. Further investigations will account for a more detailed understanding of the functions of TGase 2 in cancer.[56,62a]

TGase 2 as important mediator of biomaterial–tissue interactions: Based on its roles in the extracellular environment, another interesting research field for TGase 2 arises due to its participation in processes of tissue response to polymeric biomaterials.[64] In this regard, a number of groups have demonstrated that the crosslinking and macromolecule grafting function of TGase 2 can be applied as an exciting tool to generate crosslinked biomaterials with improved mechanical strength, resistance to proteolytic digestion, and biocompatibility.[65] On the other hand, studies on such biomaterials highlighted the importance of the adhesive properties of TGase 2 that are independent of its 'classical' crosslinking function.[66] This transamidase activity-independent promotion of cell adhesion by TGase 2 was first observed using plastic surfaces coated with native and catalytically inactive TGase 2.[67] TGase 2, when in complex with fibronectin, can enhance the attachment, spreading, viability and proliferation of cells in contact with, or migrating into, biomaterials. This, in consequence, stabilizes the implant-tissue interface and can also improve biocompatibility.[68] These effects are mediated by cell surface proteins like β1 integrins.[67] Additionally, the involvement of cell surface heparan sulfate proteoglycans has been demonstrated.[69] Human osteoblasts show increased cell spreading on poly(ε-caprolactone) when the polymer is coated with TGase 2/fibronectin compared to either uncoated or fibronectin coated materials.[70] The TGase 2/fibronectin complex binds to cell surface heparan sulfate proteoglycans such as syndecan-4 which is synthesized by osteoblasts.[71] Recently, two studies investigating the heparan sulfate-binding site on TGase 2 confirmed the importance of matrix-bound TGase 2-syndecan-4 interactions in cell adhesion.[72] Moreover, a new mechanism for rapid translocation of TGase 2 into the extracellular matrix, which involves syndecan shedding, was proposed.[72a] Other cells important in tissue regeneration like endothelial cells, smooth muscle cells, and fibroblasts also show improved attachment, spreading, and survival on synthetic degradable biomaterials in the presence of TGase 2/fibronectin. Such polymeric materials include poly(ε-caprolactone), poly(L-lactide), and poly(D,L-lactide-co-glycolide).[66,73] Future regenerative medicine will benefit from the development of comparatively simple biomaterials with extrinsic physicochemical information, which are able both to mobilize endogenous cells and to guide their development, supporting tissue reorganization. This approach will avoid the difficulties associated with culture, storage, and distribution of transplanted cells not to mention immune considerations. In this regard, a current challenge is to understand the exact role of TGase 2 in recruitment of endogenous cells into polymer films, scaffolds or particles with appropriate architecture. Furthermore, understanding the mechanisms by which TGase 2 supports subsequent tissue organization, for example, cell adhesion, differentiation, and vascularization will offer various therapeutic possibilities to

Table 1
Kinetic parameters of substrates (K_m, k_{cat} and k_{cat}/K_m) obtained in assays with human FXIII activated by thrombin

Entry	Acyl donor[a]	Acyl acceptor[a]	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	Refs.
1	**γ-Chain of fibrin**		6.2×10^{-6}	31.2	5,000,000	87a[b]
2	**H-TIGEGQQHHLGGAKQAGDV-OH**		—	—	500	87a[b]
3	**H-GQQHHLGGAKQAGDV-OH**[c]	Biotin-$H_6Q_{15}R_5$-OH	3.5×10^{-6}	0.18	50,200	111b[c]
4	**H-LGGAKQAGDV-OH**[c]	Biotin-$H_6Q_{15}R_5$-OH	4.4×10^{-6}	0.14	31,800	111b[c]
5	**H-LTIGEGQQHHLGG-OH**	[^{14}C]Dns-Cad	3.5×10^{-3}	1.55	443[d]	91d[e]
6	**γ-Chain of fibrin**	[^{14}C]Dns-Cad	4.1×10^{-5}	0.36	8780[d]	91d[e]
7	**α_{S1}-Casein**	[^{14}C]Dns-Cad	6.8×10^{-5}	0.087	1280[d]	91d[e]
8	**γ_1-Casein**	[^{14}C]Dns-Cad	2.0×10^{-5}	1.75	87,500[d]	91d[e]
9	**κ-Casein**	[^{14}C]Dns-Cad	9.2×10^{-5}	0.26	2830[d]	91d[e]
10	**β-Casein**	[^{14}C]Dns-Cad	3.1×10^{-5}	1.63	52,600[d]	91d[e]
11	**β-Casein**	H-G-OEt	4.1×10^{-5}	16.1	394,000	80c[f]
12	**H-LGPGQSKVIG-OH**	H-G-OEt	1.4×10^{-3}	78.0	54,400	80c[f]
13	**H-NQEQVSPLTLLKLGN-OH**[g]	H-G-OEt	4.6×10^{-4}	175	382,000	80c[f]
14	**H-NQEQVSPLTLLK-OH**[g]	H-G-OEt	5.3×10^{-4}	389	734,000	80a[f]
15	**Abz-NE(Cad-Dnp)EQVSPLTLLK-OH**[g]	H-G-OMe	2.0×10^{-5}	—	—	123a[h]
16	**H-YE(*p*NA)KKVIG-NH_2**	H_2O	6.5×10^{-5}	0.15	2340	119b[i]
17	**H-YE(*p*NA)VKVIG-NH_2**	H_2O	4.4×10^{-5}	0.17	3530	119b[i]
18	**H-YE(*p*NA)IKVIG-NH_2**	H_2O	7.4×10^{-5}	0.17	2320	119b[i]
19	**H-YE(*p*NA)LKVIG-NH_2**	H_2O	5.8×10^{-5}	0.12	2010	119b[i]
20	**H-FE(*p*NA)VKVIG-NH_2**	H_2O	7.4×10^{-5}	0.12	1640	119b[i]
21	**H-YE(*p*NA)VKVI-NH_2**	H_2O	9.5×10^{-5}	0.20	2060	119b[i]
22	**H-YE(*p*NA)VRVIG-NH_2**	H_2O	7.2×10^{-5}	0.16	2250	119b[i]
23	**H-YE(AMC)KKVIG-NH_2**	H_2O	6.3×10^{-5}	0.00124	19.7	121[j]
24	**H-YE(AMC)VKVIG-NH_2**	H_2O	3.9×10^{-5}	0.00103	26.4	121[j]
25	**H-YE(AMC)IKVIG-NH_2**	H_2O	3.5×10^{-5}	0.00118	33.7	121[j]
26	**H-YE(AMC)VKVI-NH_2**	H_2O	9.0×10^{-5}	0.00213	23.7	121[j]
27	**H-YE(AMC)VRVIG-NH_2**	H_2O	7.5×10^{-5}	0.00146	19.5	121[j]
28	**H-K(*N*-Me-Abz)E(Put-Dnp)VKVIG-NH_2**	H_2O	2.9×10^{-6}	0.0235	8010	121[h]
29	**H-YE(Put-Dnp)K(*N*-Me-Abz)KVIG-NH_2**	H_2O	3.7×10^{-6}	0.0177	4820	121[h]

[a] Kinetic parameters of acyl donor/acceptor substrates in bold letters.
[b] Kinetic parameters for the crosslinking of the γ-chain of fibrin or γ-chain peptide H-TIGEGQQHHLGGAKQAGDV-OH were obtained by reduced SDS–PAGE and densitometry.
[c] Oregon Green-labeled; measurement of plate-bound fluorescence (λ_{ex} = 485 nm, λ_{em} = 538 nm) in streptavidin-precoated microplates.
[d] Calculated from k_{cat} and K_m given.
[e] Measurement of protein-/peptide-incorporated radioactivity.
[f] Measurement of NADH depletion at λ = 340 nm using the GDH assay (Scheme 1).
[g] Peptide derived from the N-terminal sequence of α_2-plasmin inhibitor.
[h] FRET system: time-dependent increase in fluorescence of Abz (λ_{ex} = 313 nm, λ_{em} = 418 nm, Scheme 7) or *N*-Me-Abz (λ_{ex} = 355 nm, λ_{em} = 460 nm) due to release of the quenchers Dnp-Cad and Dnp-Put was measured, respectively. Put: putrescine.
[i] Time-dependent release of *p*-nitroaniline (*p*NA) was measured as increase in absorbance at λ = 405 nm.
[j] Time-dependent release of 7-amino-4-methylcoumarin (AMC) was measured as increase in fluorescence at λ_{ex} = 380 nm, λ_{em} = 460 nm.

stimulate wound healing and tissue regeneration during the response to artificial biocompatible and biodegradable polymers.[26a,74] Therefore, imaging of this enzyme in vivo using selective probes is desirable.

Activity assays for TGases: In the past decades, several assay methods have been developed to measure and quantify TGase activity. As outlined above, TGase activity is exhibited by a further seven isoforms, in addition to TGase 2. Most assay methods are described for FXIIIa and TGase 2 and these will be covered in this section. Due to the same type of reaction being catalyzed by different TGases, the assays are applicable in principle to several isoforms.[76] A variety of these methods are compatible with high-throughput screening and these techniques have been applied for inhibitor identification and characterization (see Table 3 and text below). TGase activity follows a ping–pong type mechanism, which includes the reversible acylation of an active-site cysteine residue (Cys277) and release of one equivalent of ammonia (in the case of a primary amide acting as acyl donor substrate, such as glutamine) prior to deacylation by hydrolysis or aminolysis (Fig. 3).[75,77] Above pH 7 aminolysis is kinetically favored over hydrolysis, while hydrolysis becomes more important at pHs below 7.[78] Both the acyl donor and acyl acceptor substrates, as well as products formed in the transamidation reaction, have been used to quantify the enzymatic activity. Kinetic parameters for selected substrates of human FXIIIa and guinea pig TGase 2, that is, the Michaelis constant, K_m, the first order catalytic rate constant, k_{cat}, and the apparent second order rate constant k_{cat}/K_m, are summarized in Tables 1 and 2.

a) Assays for determining the product ammonia: One of the best established TGase assay strategies is a glutamate dehydrogenase (GDH)-coupled method (Scheme 1) allowing for the continuous monitoring of TGase-catalyzed release of ammonia.[77,79] This assay is applicable if either or both the acyl-acceptor and acyl donor substrate are proteins or small molecules/oligopeptides (Table 1, entries 11–14; Table 2, entries 1, 2, 6, 7, 9, 10).[77,80] Addition of GDH, α-ketoglutarate and either NADH[77] or NADPH[79] to the TGase assay mixture results in a decrease in absorbance at 340 nm, due to the oxidation of NAD(P)H to $NAD(P)^+$. Kárpáti et al.[80a] increased the sensitivity of the coupled assay for quantification of FXIIIa activity by exchanging NADPH for NADH, introducing the dodecapeptide H-NQEQVSPLTLLK-OH derived from the N-terminal sequence of α_2-plasmin inhibitor as new acyl donor substrate, optimizing the concentration of assay components and using an appropriate blank sample (Table 1, entry 14). Further assay optimization was done by Kappel et al.,[81] who applied thio-NADH (Scheme 1) as the GDH substrate, allowing for detection of FXIIIa activity at 405 nm with a concomitant increase in trueness of measurement and assay robustness compared to the NADH-based methods. In order to obtain reliable results in the GDH-coupled assay, enzyme amounts have to be adjusted in a way that ensures the TGase reaction to be rate-limiting ([TGase] << [GDH]). Furthermore, it is necessary to prove that neither

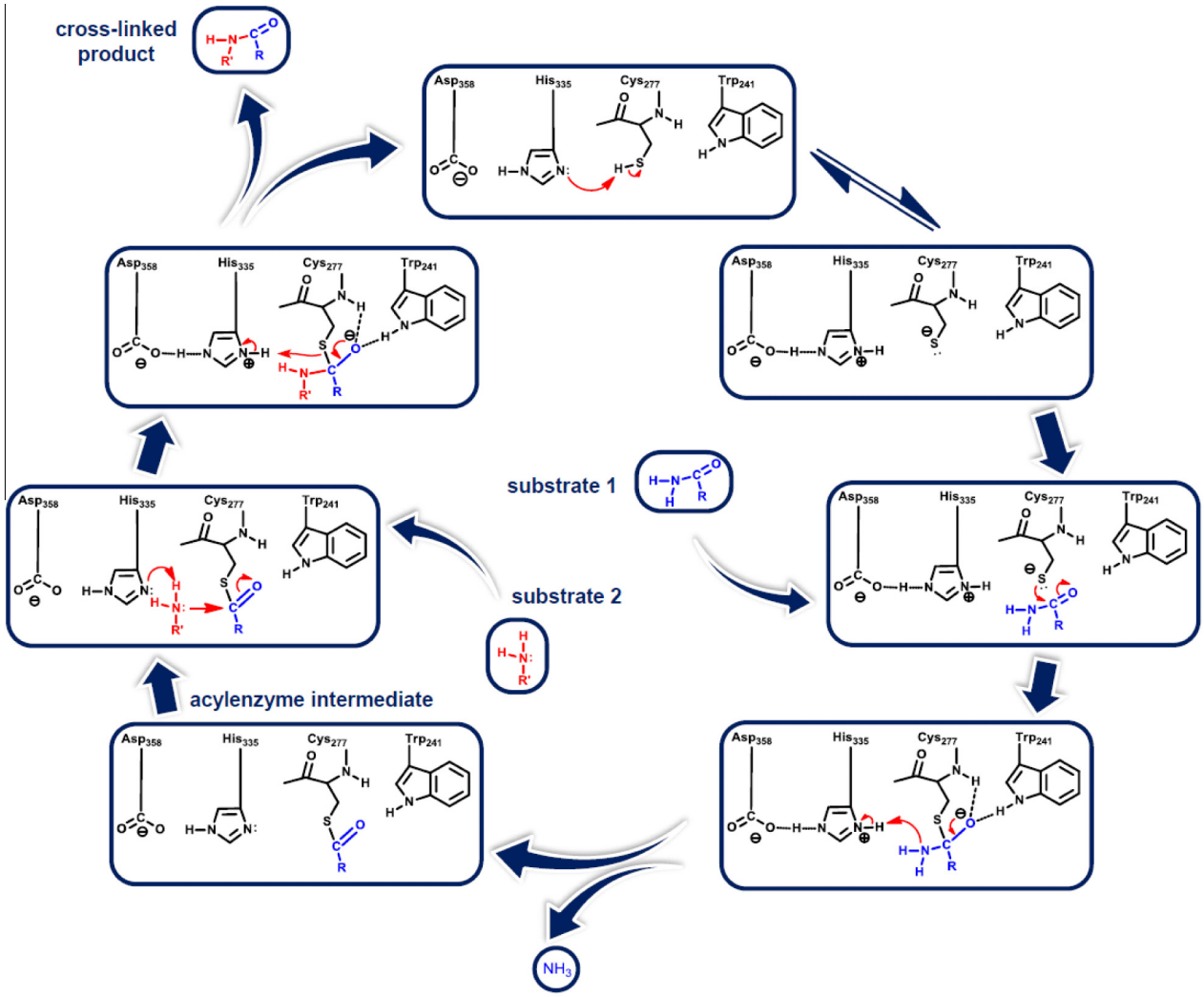


Figure 3. Schematic representation of the mechanism of TGase 2 transamidase activity. Acyl transfer to water and alcohols obeys a similar mechanism. Residue numbering corresponds to the human enzyme. Created according to a scheme of Iismaa et al.[75]

substrates nor products of one enzyme interfere with the activity of the other enzyme, and that GDH is no substrate of the TGase.[77] GDH-coupled TGase assays have been developed for the accurate quantification of FXIIIa activity in plasma samples, which is necessary before and during replacement therapy of patients suffering from low FXIIIa plasma levels.[80a,81] Moreover, as the GDH-coupled method is applicable to a wide range of acyl donor and acyl acceptor substrates, provided that the former contains a primary amide group, it represents a useful tool for general TGase kinetic studies, including characterization of substrates[77,80c] and inhibitors (Table 3, entry 1).[80b,82] However, as this assay is rather insensitive (TGase concentrations often used in inhibition assays are in the high nanomolar range),[80b,83] it is not suitable to investigate highly potent inhibitors.[82d] Although the GDH-coupled assay is frequently encountered in the literature, the majority of techniques for monitoring TGase activity are based on other detection methods.

Another assay strategy described by Flanagan and FitzGerald[84] uses *o*-phthaldialdehyde (OPA) for the discontinuous quantification of ammonia release during TGase-catalyzed cross-linking of sodium caseinate. Here, the formed ammonia is spectrophotometrically quantified in the supernatant at 340 nm after protein precipitation with trichloroacetic acid (TCA). Alternatively, a strategy which has become the preferred method for determining the degree of cross-linkage in food proteins catalyzed by microbial TGases[85] was developed by Church et al.[86] and involves the OPA reagent being used to determine protein cross-linking, by following the decrease of OPA-reactive protein-bound amino groups. However, this method is claimed to be less feasible than that of Flanagan and Fitzgerald 'due to the potential concealment of amino groups within cross-linked sodium caseinate'.[84]

b) Assays using proteins as both acyl donor and acyl acceptor substrates: A range of discontinuous assay strategies have been developed to monitor the TGase-catalyzed cross-linking of proteins, that is, by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE, Table 1, entry 1),[87] ion exchange chromatography (quantification of the cross-linking level),[88] and an enzyme-linked immunosorbent assay (ELISA)-like procedure.[88b] The cross-linking of proteins has been used to investigate the kinetics of the reaction of FXIIIa with the fibrin γ-chain[87a] and to develop a transglutaminase activity staining for histological tissue sections, using His_6-Xpress-tagged green fluorescent protein (GFP) as a substrate.[87b]

In order to design new peptidyl linkers for the site-specific enzymatic conjugation of functional proteins, the substrate specificity of microbial TGases has recently been investigated with two fluorescence resonance energy transfer (FRET)-based strategies on fluorescent model proteins. On the one hand, the increase in fluorescence of the FRET acceptor protein is (continuously) fol-

Table 2
Kinetic parameters of substrates (K_m, k_{cat} and k_{cat}/K_m) obtained in assays with guinea pig TGase

Entry	Acyl donor[a]	Acyl acceptor[a]	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	Refs.
1	***N,N*-Dimethylcasein**		3.3×10^{-6}	0.14	44,000	80b[b]
2	***N,N*-Dimethylcasein**	H-G-OMe	1.6×10^{-6}	0.16	97,000	80b[b]
3	***N,N*-Dimethylcasein**	Dns-Cad	2.3×10^{-6}	0.14	64,000	80b[c]
4	***N,N*-Dimethylcasein**	Boc-K-NH$(CH_2)_2$NH-Dns	1.5×10^{-6}	0.095	65,000	101[c]
5	**Cbz-QG-OH**	$HONH_2$	6.7×10^{-2}	48.8	728	91a,105b[d]
6	**Cbz-QG-OH**	Ac-K-OMe	9.6×10^{-3}	2.80	292[e]	77[b]
7	**Cbz-QG-OH**	CH_3NH_2	1.3×10^{-2}	3.07	236[e]	77[b]
8	**Cbz-QG-OH**	DMPDA	5×10^{-3}	1	200[e]	115b[f,g]
9	**Cbz-QG-OH**	H_2O	1.8×10^{-3}	0.17	96	80b[b]
10	**Cbz-QG-OH**	H-G-OMe	1.4×10^{-3}	0.16	110	80b[b]
11	**Cbz-QG-Cad-Dns**	$CH_3(CH_2)_3NH_2$	9×10^{-4}	—[h]	—	112a[i,j]
12	**Cbz-L-E(ONp)G-OH**	H_2O	8.7×10^{-6}	0.83	95,400[e]	118a[k]
13	**Cbz-D-E(ONp)G-OH**	H_2O	2.5×10^{-4}	0.91	3640[e]	118a[k]
14	**Cbz-G-γAbu-OCou**	H_2O	9.0×10^{-6}	1.25	139,000[e]	120a[l]
15	**Cbz-F-γAbu-OCou**	H_2O	7.0×10^{-6}	0.75	107,000[e]	120a[l]
16	**Cbz-F-γAbu-OCou**	$NCCH_2NH_2$	2.5×10^{-5}	2.23	89,200[e]	120a[l]
17	**Cbz-F-γAbu-OCou**	Ac-K-OMe	1.5×10^{-5}	1.85	123,000[e]	120a[l]
18	Casein	**Biotin-TVQQEL-OH**	1×10^{-6}	—	—	93c[m]
19	*N,N*-Dimethylcasein	**Biotin-Cad**	5.3×10^{-5}	—	—	97a[n]
20	*N,N*-Dimethylcasein	**Dns-Cad**	1.4×10^{-5}	—	—	99[o]
21	5-Fam-HQSYVDPWMLDH-OH	**BSA**	3.7×10^{-6}	—	—	104[p,q]
22	(β-Phenylpropionyl)thiocholine	**Dns-Cad**	3×10^{-7}[r]	0.8	2,670,000[e]	91c[s]
23	(β-Phenylpropionyl)thiocholine	**Dns-thia-Cad**	7×10^{-7}[r]	0.8	1,140,000[e]	91c[s]
24	Cbz-QG-OH	**DMPDA**	2.5×10^{-4}	—	—	115a[g]
25	Cbz-QG-Cad-Dns	**$CH_3(CH_2)_3NH_2$**	9.9×10^{-4}	5.52	5570	112a[i,j]
26	Cbz-F-γAbu-OCou	**$NCCH_2NH_2$**	1.6×10^{-5}	1.23	76,900[e]	120a[l]
27	Cbz-L-E(ONp)G-OH[t]	**$NCCH_2NH_2$**	9.8×10^{-5}	1.9	19,100	118b[k]
28	Cbz-F-γAbu-OCou	**Ac-K-OMe**	1.5×10^{-3}	1.6	1070[e]	120a[l]
29	Cbz-L-E(ONp)G-OH[t]	**Ac-K-OMe**	2.8×10^{-3}	0.47	167	118b[k]
30	Cbz-G-OCou	**H-G-NH_2**	7.0×10^{-3}	0.67	95.7[e]	120b[l]
31	Cbz-L-E(ONp)G-OH[t]	**H-G-NH_2**	4.7×10^{-3}	1.75	373	118b[k]

[a] Kinetic parameters of acyl donor/acceptor substrates in bold letters.
[b] Measurement of NADH depletion at $\lambda = 340$ nm using the GDH assay (Scheme 1).
[c] Increase in fluorescence due to crosslinking of Dns-Cad (Scheme 2, $\lambda_{ex} = 330$ nm, $\lambda_{em} = 500$ nm) and Boc-K-NH$(CH_2)_2$NH-Dns ($\lambda_{ex} = 330$ nm, $\lambda_{em} = 530$ nm) with *N,N*-dimethylcasein was measured, respectively.
[d] Quantification of formed hydroxamate by measurement of absorbance at $\lambda = 525$ nm after addition of $FeCl_3$-TCA reagent.[105a]
[e] Calculated from k_{cat} and K_m given.
[f] His_6-gpTGase.
[g] Time-dependent product formation (Cbz-E(DMPDA)G-OH, Scheme 5) was measured as increase in absorbance at $\lambda = 278$ nm.
[h] $V_{max} = 8.4$ μmol min^{-1} mg^{-1}.
[i] TGase from bacterium *Streptoverticillium mobaraense*.
[j] HPLC-based assay. Product formation was monitored by measurement of both absorbance at $\lambda = 280$ nm and fluorescence at $\lambda_{ex} = 330$ nm, $\lambda_{em} = 520$ nm.
[k] Time-dependent release of *p*-nitrophenol (HONp) was measured as increase in absorbance at $\lambda = 400$ nm (Scheme 6).
[l] Time-dependent release of 7-hydroxycoumarin (HOCou) was measured as increase in fluorescence at $\lambda_{ex} = 330$ nm, $\lambda_{em} = 460$ nm (Scheme 6). γAbu: γ-aminobutyric acid.
[m] Crosslinking of Biotin-TVQQEL-OH with casein was quantified using ExtrAvidin-peroxidase, H_2O_2 and tetramethylbenzidine (TMB). After stopping the peroxidase reaction with H_2SO_4, absorbance was measured at $\lambda = 450$ nm.
[n] Crosslinking of Biotin-Cad with *N,N*-dimethylcasein was quantified with streptavidin-β-galactosidase and *p*-nitrophenyl-β-galactopyranoside. *p*-Nitrophenol formation was quantified at $\lambda = 405$ nm.
[o] Fluorescence of Dns-Cad (Scheme 2, $\lambda_{ex} = 340$ nm, $\lambda_{em} = 535$ nm) crosslinked to *N,N*-dimethylcasein was measured.
[p] Recombinant hTGase 2.
[q] Time-dependent crosslinking of 5-Fam-HQSYVDPWMLDH-OH to BSA was measured as increase in fluorescence anisotropy at $\lambda_{ex} = 485$ nm, $\lambda_{em} = 535$ nm (Scheme 3).
[r] K_m values were corrected for the 'effective concentrations of unprotonated amine species'. Uncorrected K_m values for Dns-Cad and Dns-thio-Cad were 3×10^{-4} M ($k_{cat}/K_m = 2670$ M^{-1} s^{-1})[e] and 7×10^{-5} M ($k_{cat}/K_m = 11{,}400$ M^{-1} s^{-1})[e], respectively.[91c]
[s] Measurement of fluorescence ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 460$ nm) of the amide coupling products *N*-(β-phenylpropionyl)dansylcadaverine and *N*-(β-phenylpropionyl)dansylthiacadaverine, respectively (Scheme 4).[113b]
[t] Kinetic parameters for hydrolysis of Cbz-L-E(ONp)G-OH: $K_m = 2.0 \times 10^{-5}$ M; $k_{cat} = 0.28$ s^{-1}, $k_{cat}/K_m = 14{,}200$ M^{-1} s^{-1}.

lowed in vitro using acyl acceptor peptide- and acyl donor peptide-tagged fluorescent protein pairs, such as enhanced blue fluorescent protein/enhanced GFP (eBFP/eGFP) and enhanced cyan fluorescent protein/enhanced yellow fluorescent protein (eCFP/eYFP).[89] On the other hand, both in vitro and in vivo quenching assays have been developed, monitoring the decrease of yellow fluorescence of the FRET donor protein eYFP (covalently attached to the acyl acceptor substrate) when crosslinked to *Discosoma* sp. red fluorescent protein (DsRed, covalently attached to the acyl donor substrate). In this system, the fluorescence of the FRET acceptor DsRed does not significantly increase due to the TGase-catalyzed reaction.[90]

c) Assays using a combination of proteins and small molecules/oligopeptides as acyl donor and acyl acceptor substrates: A large variety of discontinuous and continuous assays follows the TGase-catalyzed incorporation of small molecules/oligopeptides into proteins by quantification of the macromolecular product. Discontinuous methods include the reaction of ^{14}C- and ^{3}H-labeled primary amines (e.g., [^{14}C]monodansylcadaverine {[^{14}C]Dns-Cad} and both [^{14}C]- and [^{3}H]putrescine) with various types of casein and the measurement of the protein-bound radioactivity after TCA precipitation (Table 1, entries 6–10).[91] In the past, these radiometric assays have often been used for the characterization of TGase inhibitors (Table 3, entry 2),[91a,92] and for the quantification of TGase activity in biological samples. The latter includes the measurement of FXIII activation in human plasma[91e] and determination of TGase 2 activity in human brain samples of patients suffering from Alzheimer's disease (AD).[91f] Another assay strategy monitors the incorporation of biotinylated glutamine- or

Table 3
TGase 2 assays used for the identification and characterization of inhibitors

Entry	Acyl donor	Acyl acceptor	Mode	Refs.
1	Cbz-QG-OH	H_2O	Continuous	82[a]
2	Casein	[^{14}C]- and [^{3}H]putrescine	Discontinuous	91a,92[b]
3	*N*,*N*-Dimethylcasein	Biotin-Cad	Discontinuous	95,96,126[c]
4	*N*,*N*-Dimethylcasein	Dns-Cad	Continuous	102a–c,102h[d]
5	*N*,*N*-Dimethylcasein	Boc-K-NH$(CH_2)_2$NH-Dns	Continuous	95,102d–g[e]
6	Cbz-QG-OH	$HONH_2$	Discontinuous	105b,108,109[f]
7	Cbz-QG-OH	DMPDA	Continuous	116[g]
8	Cbz-E(ONp)G-OH	H_2O	Continuous	127[h]
9	Cbz-F-γAbu-OCou	H_2O	Continuous	82d,127a[i]
10	Dns-ε-aminocaproyl-QQIV-OH	Dnp-Cad	Continuous	102h[j]
11	Abz-APE(Cad-Dnp)QEA-OH	H-G-OMe	Continuous	128[k]

[a] Measurement of NADH depletion at λ = 340 nm using the GDH assay (Scheme 1).
[b] Scintillation counting of ^{3}H incorporated into TCA-precipitated casein.
[c] Determination of the increase in absorbance at λ = 405 nm due to nitrophenyl phosphate hydrolysis catalyzed by streptavidin-conjugated alkaline phosphatase.[93a]
[d] Increase in fluorescence due to cross-linking of Dns-Cad (Scheme 2, λ_{ex} = 360 nm, λ_{em} = 500 nm) with *N*,*N*-dimethylcasein was measured.[102a]
[e] Increase in fluorescence due to cross-linking of Boc-K-NH$(CH_2)_2$NH-Dns (λ_{ex} = 350 nm, λ_{em} = 535 nm) with *N*,*N*-dimethylcasein was measured.[76]
[f] Quantification of formed hydroxamate by measurement of absorbance at λ = 525 nm after addition of $FeCl_3$-TCA reagent.[105a]
[g] Product formation (Cbz-E(DMPDA)G-OH, Scheme 5) was measured as increase in absorbance at λ = 278 nm.[115a]
[h] Release of HONp was measured as increase in absorbance at λ = 400 nm (Scheme 6).[118b]
[i] Release of HOCou was measured as increase in fluorescence at λ_{ex} = 330 nm, λ_{em} = 460 nm (Scheme 6).[120a]
[j] FRET system: decrease in fluorescence due to quenching of Dns-ε-aminocaproyl-QQIV-OH (λ_{ex} = 360 nm, λ_{em} = 590 nm) by the quencher Dnp-Cad.[129]
[k] FRET system: time-dependent increase in fluorescence of Abz (λ_{ex} = 313 nm, λ_{em} = 418 nm) due to release of the quencher Dnp-Cad was measured.[123b]

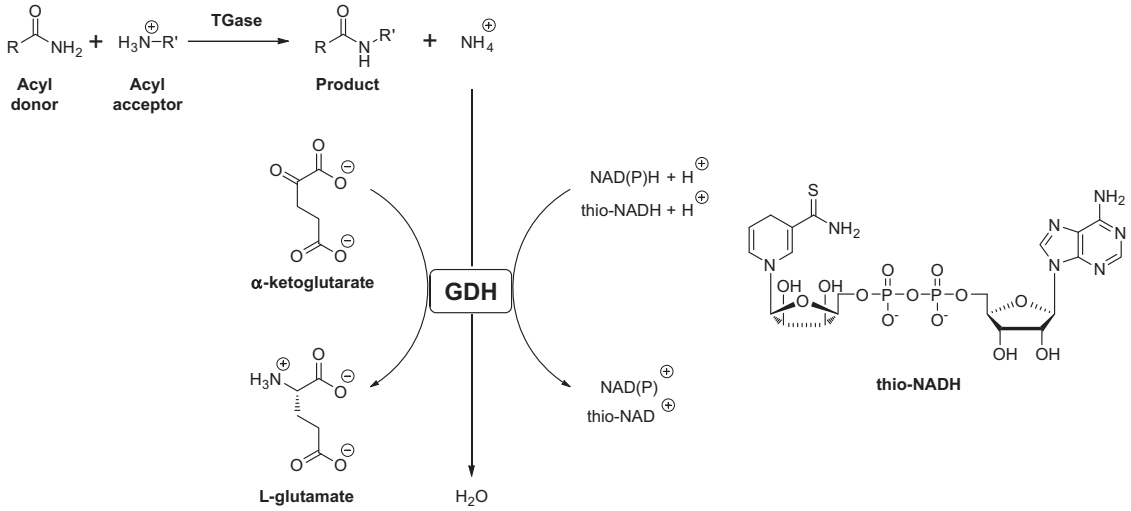
amine-containing substrates into immobilized proteins, such as sodium caseinate, *N*,*N*-dimethylcasein and fibrinogen. For product detection, colorimetric assays using avidin-/streptavidin-enzyme conjugates (Table 2, entry 18)[93] or fluorophore-labeled streptavidin[94] are applied. This assay strategy has often been used for screening and characterization of TGase inhibitors (Table 3, entry 3)[95,96] and was applied for the determination of FXIIIa in human plasma[93b] and the simultaneous measurement of TGase 2 and FXIIIa activities in human cell extracts.[94] A similar method has been employed to investigate in situ TGase activity after immobilization of the biotinylated products (Table 2, entry 19) and to study the cellular regulation of the enzyme.[97]

The application of discontinuous assay strategies to kinetic analysis of enzymatic reactions requires sampling of the reaction mixture at time intervals with both control of sample volume and separation of substrate and product being absolutely necessary. In contrast, continuous assay methods allow for a relatively simple real-time measurement of enzyme activity.[98] Some of these continuous methods are based on an observation by Lorand et al.,[98a] who found the TGase-catalyzed incorporation of Dns-Cad into α-casein (Scheme 2) to result in a 'shift in both the wavelength (535 nm to ~515 nm) and the intensity of fluorescence of the dansyl group' due to placement of the fluorophore in a more hydrophobic environment (Table 2, entry 3).[80b]

The same principle is applied in the reaction of *N*,*N*-dimethylcasein with Boc-K-NH$(CH_2)_2$NH-Dns (Table 2, entry 4).[101] Both methods have been extensively used in the search for inhibitors (Table 3, entries 4 and 5).[76,95,102] Another continuous assay strategy utilizes the increase in fluorescence anisotropy[103] due to incorporation of fluorescent molecules, such as Dns-Cad[100] (acyl acceptor substrate, Scheme 2) or 5-carboxyfluorescein (5-Fam)-labeled PepT26 (acyl donor substrate, Scheme 3; Table 2, entry 21)[104] into Hammarsten casein and bovine serum albumin (BSA), respectively. The latter method is particularly sensitive (picomolar concentrations of TGase are detected) and can be easily adapted for the high-throughput screening of TGase inhibitors.[104]

d) Assays using small molecules/oligopeptides as both acyl donor and acyl acceptor substrates: Both discontinuous and continuous TGase assay methods have also been developed with low-molecular weight compounds/oligopeptides as acyl donor and acyl acceptor substrates. One of the earliest methods monitors the reaction between Cbz-QG-OH and hydroxylamine (Table 2, entry 5),[91a,105] with the hydroxamate formed being spectrophotometrically quantified after addition of $FeCl_3$-TCA reagent[106] and removal of the precipitated protein.[107] Although relatively insensitive (see below, micromolar concentrations of TGase are used in the assay),[108] this method has been applied in the past for inhibitor identification (Table 3, entry 6).[105b,108,109] Other discontinuous assay strategies include separation of substrate from product followed by ELISA-like procedures[110] or densitometric (Table 1, entry 2),[87a] radiometric (Table 1, entry 5),[91d] and fluorescence-based (Table 1, entries 3 and 4)[111] product detection, respectively. Recently, two fluorimetric HPLC-based techniques have been introduced which monitor the coupling of Cbz-QG-OH to Dns-Cad and that of Cbz-QG-Cad-Dns to unlabeled amines, such as butylamine (Table 2, entries 11 and 25), with the latter one having been used for the characterization of TGase acyl acceptor substrates and TGase preparations.[112]

Continuous assay strategies with small molecules as substrates often feature spectrophotometric, fluorimetric or FRET-based readouts, which allows for the kinetic analysis of the enzymatic reaction. An early method introduced by Lorand[113] applies a two-phase system (water/*n*-heptane) using (β-phenylpropionyl)-thiocholine and Dns-Cad/dansylthiacadaverine (Dns-thia-Cad) as water soluble acyl donor and acyl acceptor substrates, respectively (Scheme 4, Table 2, entries 22 and 23).[91c] The hydrophobic coupling products, that is, *N*-(β-phenylpropionyl)dansylcadaverine or *N*-(β-phenylpropionyl)dansylthiacadaverine, are continuously extracted into the *n*-heptane phase and quantified by direct fluorescence measurement.[113b] The same substrates have also been used in an aqueous assay system, with the formation of the second product, that is, thiocholine, being continuously determined with Ellman's reagent.[114] Both methods were shown to give comparable kinetic parameters K_m and k_{cat} for the two amine substrates.[113b,114] Another homogenous assay monitors the product formation of the reaction between Cbz-QG-OH and *N*,*N*-dimethyl-1,4-phenylenediamine (DMPDA) (Scheme 5; Table 2, entries 8 and 24),[115] and has been used for characterization of some TGase inhibitors (Table 3, entry 7).[116] A fluorescence-based strategy follows the transamidation between Cbz-QG-Cad-Dns and Dns-Cad to give highly fluorescent Cbz-E(Cad-Dns)G-Cad-Dns.[117] The two substrates of this assay are both dansylated, which increases their affinity for



Scheme 1. Glutamate dehydrogenase (GDH)-coupled TGase assay. Ammonium produced by the TGase reaction is used for the GDH-catalyzed reductive amination of α-ketoglutarate to L-glutamate.[77,79]

TGase, while π–π stacking of the two dansyl groups within the product results in fluorescence enhancement.[91c,117]

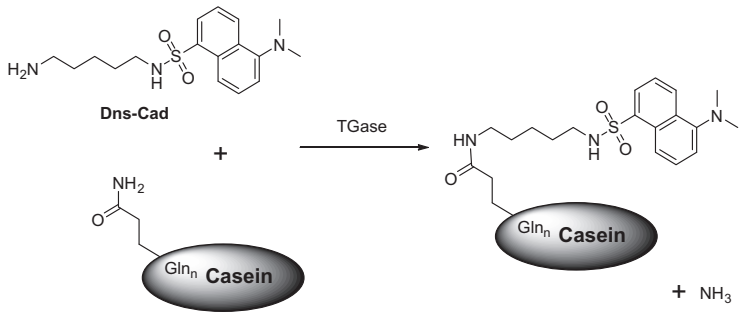
Another category of TGase assays monitor the hydrolysis/aminolysis of chromogenic and fluorogenic acyl donor substrates by determining the release of the chromophores *p*-nitrophenol (HONp, Scheme 6 and Table 2, entries 12, 13, 27, 29 and 31)[118] and *p*-nitroaniline (*p*NA, Table 1, entries 16–22)[119] or the fluorophores 7-hydroxycoumarin (HOCou, Scheme 6 and Table 2, entries 14–17, 26, 28, 30)[120] and 7-amino-4-methylcoumarin (AMC, Table 1, entries 23–27).[121]

Recently, application of small molecule substrates has been described for FRET-based TGase assays. Gnaccarini et al.[122] use a strategy that follows the ligation-dependent decrease in fluorescence of the FRET donor group (coumarin), upon excitation at $\lambda = 405$ nm, as the donor group fluorescence is more pronounced than the concomitant increase of the FRET acceptor fluorescence (fluorescein). Similarly, decrease of Dns fluorescence in Dns-ε-aminocaproyl-QQIV-OH occurs due to reaction with the quencher Dnp-Cad (Table 3, entry 10).[2b] Other methods, in contrast, apply internally quenched FRET systems as acyl donor substrates, such as 2-(methylamino)benzoyl (*N*-Me-Abz)/dinitrophenyl (Dnp) (Table 1, entries 28 and 29)[121] and 2-aminobenzoyl (Abz)/Dnp (Scheme 7; Table 1, entry 15 and Table 3, entry 11).[123] Here, release of the quencher Dnp during the TGase-catalyzed transamidation results in the increase of fluorescence of *N*-Me-Abz and Abz, respectively.[121,123] As reflected by entries 8–11 in Table 3, the assay strategies outlined in the previous and present paragraphs have been successfully employed for the identification and kinetic characterization of TGase inhibitors.

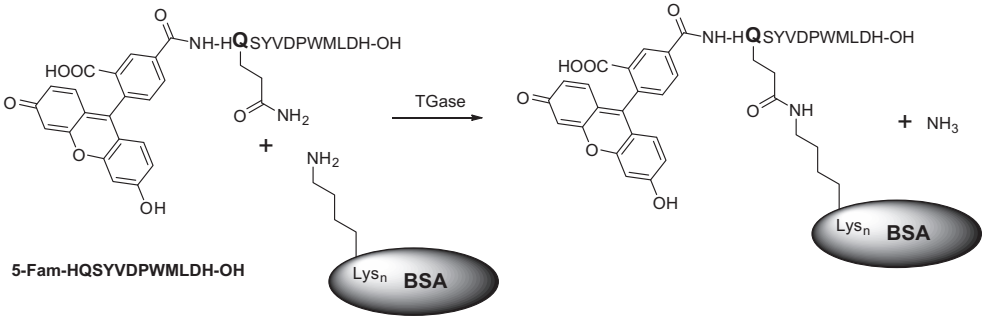
The various TGase assay methods described in the literature have been shown to have their advantages and disadvantages. The hydroxamate assay, using Cbz-QG-OH and hydroxylamine is often applied to determine the specific activity of TGase. Although fast and reproducible, this method is relatively insensitive as the extinction coefficient of the formed hydroxamate-Fe^{3+} complex is relatively small (850 M^{-1} cm^{-1} at $\lambda_{max} = 525$ nm) and thus comparatively large amounts of enzyme are required to obtain accurate results.[91a,107,112b,117,124] ELISA-like assay procedures are more sensitive, but contain several steps for quantification of enzyme activity.[117] Radiometric and fluorimetric assays remain the most sensitive and accurate methods for determining TGase activity.[110a,125] However, techniques using radioactively labeled substrates require special facilities and are relatively time consuming, which limits their use for investigating large numbers of compounds.[88b,117,125] The different fluorescent assays available also have certain limitations. Separation of conjugated products from fluorescently-labeled reactants is a common problem encountered with discontinuous assays, and may be necessary for continuous methods, e.g., the method of Lorand et al.[98a] (Scheme 2) monitors 'relative fluorescence enhancement', as free fluorescent substrate molecules interfere with product fluorescence.[99,110a] Another continuous fluorimetric method introduced by this group applies a two-phase system, by which fluorescent product and substrate are separated.[113] Here, the nature of the assay prevents its use for high-throughput inhibitor screening.[117] The assay by Jeitner et al.[117] allows for the direct continuous fluorimetric determination of the TGase-mediated transamidation by quantification of the product Cbz-E(Cad-Dns)G-Cad-Dns.[120a] While this assay is one of the most sensitive methods, it is very specific for the two substrates used, that is, Cbz-QG-Cad-Dns and Dns-Cad (see above). In contrast, fluorogenic acyl donor substrates releasing 7-hydroxycoumarin during acyl-enzyme formation (Scheme 6) can be utilized for characterization of various acyl acceptor substrates.[120] These fluorogenic acyl donor substrates show minimal nonenzymatic hydrolysis and thus are more suitable than the similarly used chromogenic *p*-nitrophenyl esters, such as Cbz-E(ONp)G-OH (Scheme 6).[111a,118b,120a] Although valuable tools, the applicability of such fluorogenic substrates is limited due to their poor solubility under assay conditions.[120a] In contrast, fluorogenic FXIIIa substrates releasing AMC (Table 1, entries 23–27) are less suited for activity measurements than their corresponding chromogenic *p*NA analogs (Table 1, entries 16–18, 21 and 22). Although having similar K_m values, the AMC substrates are hydrolyzed with k_{cat} values that are two orders of magnitude lower than those of the *p*NA derivatives, which has been attributed to the bulkiness of the coumarin ring system.[119b,121] A complementary assay strategy allowing for the characterization of acyl donor substrates is the DMPDA method (Scheme 5), which monitors the transamidase activity of TGase by spectrophotometric determination of the formed anilide.[111a,115]

The acyl donor/acyl acceptor pairs often employed for the development of TGase 2 inhibitors are surveyed in Table 3. Continuous assays are preferably applied for this purpose, with *N*,*N*-dimethylcasein being the most abundantly used acyl donor substrate.

Recent developments toward TGase 2 inhibitors: As mentioned earlier, TGase 2 represents a multifunctional enzyme. Amongst



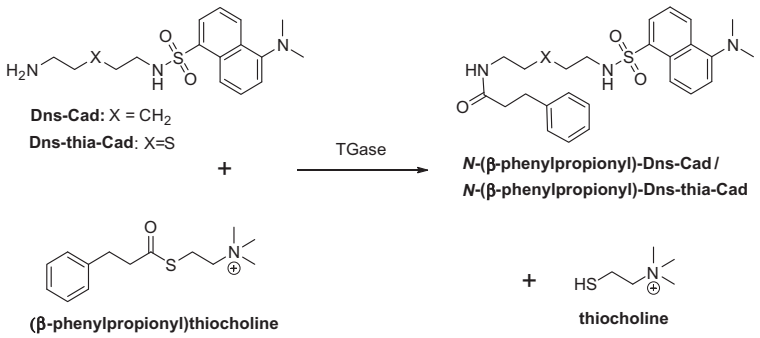
Scheme 2. TGase-catalyzed crosslinking of dansylcadaverin (Dns-Cad) to casein. Various detection methods for quantification of the macromolecular product have recently been applied, such as measurement of protein-bound Dns fluorescence (Table 2, entry 20),[99] increase in Dns fluorescence,[80b,98a] and fluorescence anisotropy[100] or protein-bound radioactivity (use of [^{14}C]Dns-Cad).[91d]



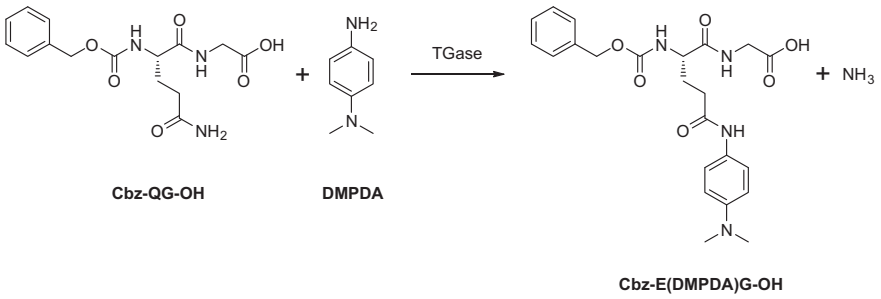
Scheme 3. Fluorescence anisotropy-based TGase assay. Incorporation of 5-carboxyfluorescein (5-Fam)-labeled peptide 'PepT26' (sequence H-HQSYVDPWMLDH-OH) into BSA is quantified as increase in fluorescence anisotropy at λ_{ex} = 485 nm, λ_{em} = 535 nm.[104]

the different enzymatic activities, the transamidase and GTPase activities are the ones best confirmed experimentally. As a result, inhibitors for this enzyme can be categorized into those targeting the transamidase or acyltransferase domain and those binding to the GTPase domain. As compounds of the latter category mostly represent analogs of GTP and GDP, which stabilize the closed, transamidase-inactive conformation, they concomitantly act as uncompetitive inhibitors of the transamidase domain. Inhibitors that target the transamidase domain directly are very often substrate analogs containing an electrophilic group (also referred to as a 'warhead') which results in an irreversible covalent interaction with the active-site thiol of Cys277. The classes of TGase 2 inhibiting compounds have recently been reviewed in depth.[39,130] More recent publications of inhibitors describe compounds with an acrylamide moiety as warhead, which were developed as therapeutic agents against Huntington's disease (HD) (Table 4).[102e–g]

In one of these studies, a collection of 283,000 compounds was screened resulting in four confirmed hits. Out of these, *N*-4-bromophenyl acrylamide was rated the most attractive lead structure. Substitution of the bromine atom by a piperazine-1-sulfonyl moiety enabled extensive structure–activity relationship (SAR) studies by attaching various acyl residues to the piperazine-N4-atom. Compound **1** was found to be the most potent TGase 2 inhibitor of this series, based on the IC_{50} value displayed upon 30 min preincubation of the compound with the enzyme monitored using the fluorimetric assay of Case et al. outlined above.[76,101] The adamantyl moiety of **1** is thought to interact with a lipophilic region defined by Phe316, Leu312, Ile331, and Leu420 (hTGase 2 numbering). This binding pocket seems to be important for the selectivity over other TGase isoforms, as it consists of more polar side chains within the latter enzymes. The importance of the acrylamide and sulfonyl groups was also confirmed within this series of compounds. For a



Scheme 4. TGase-catalyzed amide formation between Dns-Cad/Dns-thia-Cad and (β-phenylpropionyl)thiocholine. TGase activity is quantified by continuous extraction of crosslinked product in *n*-heptane and measurement of fluorescence[113b] or by determination of released thiocholine with Ellmann's reagent (5,5′-dithiobis-(2-nitrobenzoic acid)).[114]



Scheme 5. TGase-catalyzed DMPDA assay, that is, coupling of Cbz-QG-OH with *N*,*N*-dimethyl-1,4-phenylenediamine (DMPDA). Formation of the anilide product Cbz-E(DMPDA)G-OH is followed by measuring the absorbance at $\lambda = 278$ nm.[115a]

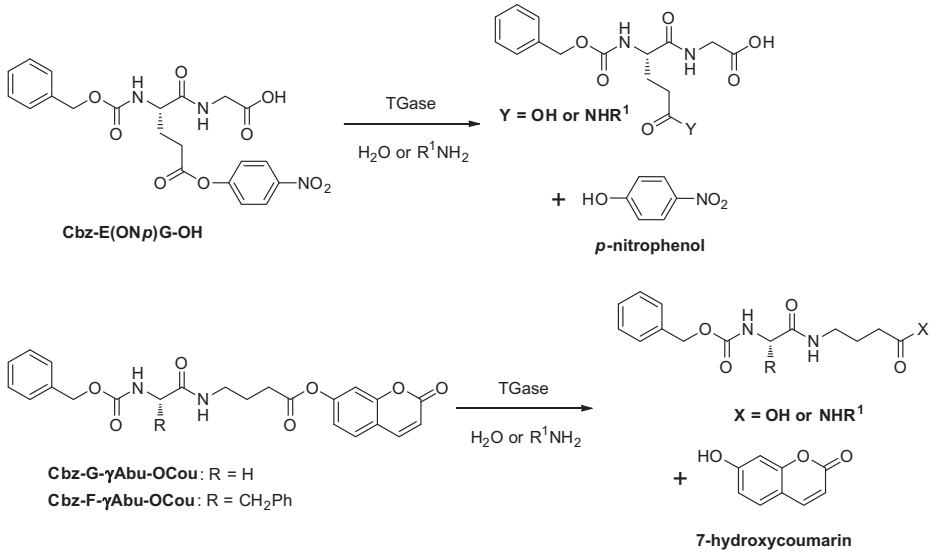
limited selection of inhibitors, drug metabolism and pharmacokinetics (DMPK) profiling was performed examining the plasma protein binding, membrane permeability and plasma stability among other parameters. The analysis revealed rather short half-lifes in mouse plasma for this inhibitor type (248 min for compound **1**), which was attributed to hydrolytic cleavage of the acrylamide C–N bond.[102e] Therefore, the aromatic and cycloaliphatic moieties were inverted for the next generation of nonpeptidic acrylamide inhibitors by attaching the acryloyl residue to 4-aminopiperidine instead of aniline. Comprehensive investigation of SAR for the substituent at the 4-position of the benzenesulfonyl moiety resulted in compound **2** (Table 4) which is ten times less potent at inhibiting hTGase 2 than **1** but exhibits a considerably increased plasma half-life.[102f]

SAR were also explored for a series of related compounds consisting of N^{α}-acyl-N^{ε}-acryloyl-lysine-amides.[102g] The inhibitory properties of this structural scaffold were earlier discovered by Marrano et al.[116b] Attachment of a 4-arylpiperazinyl moiety to the lysine carboxy group was revealed to be advantageous for the inhibitory potency. The most potent compound of this series, that is, compound **3** (Table 4), displayed an IC_{50} value as low as 14 nM together with an excellent selectivity profile for hTGase 2 over other TGase isoforms. In addition, inhibitor **3** exhibited high stability in mouse and human plasma with half-lifes of more than 24 h.[102g]

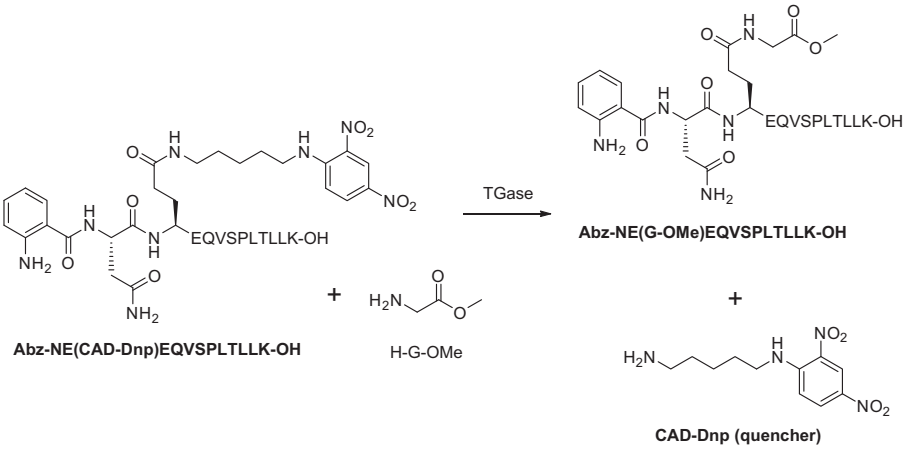
All acrylamides discussed have been found to undergo active cellular efflux mediated by P-glycoprotein. Therefore, the authors evaluated them to have limited suitability for targeting TGase 2 in CNS-related disorders.

Inhibitors bearing Michael acceptors are also the subject of a patent, published in 2008 by Oertel.[128] The glutamine residue of peptidic substrates was replaced by a moiety derived from (*S*)-2-amino-5-hexenoic acid containing electron-withdrawing substituents, such as alkoxycarbonyl and methylsulfonyl in 6-position. This places the electrophilic site prone to nucleophilic attack by the thiol group of Cys277 at exactly the same position as the side-chain carbonyl of glutamine. The IC_{50} values of 54 compounds of this type for inhibition of hTGase 2, hTGase 1 and FXIIIa are reported and the three most potent inhibitors are shown in Table 5. Compounds **4**–**6** display potent and selective inhibition of hTGase 2 over FXIIIa. X-ray crystal structures of hTGase 2 in complex with the free carboxylic acids derived from the methyl esters **4** (3S3P) and **5** (3S3S) have been solved and deposited in the Protein Data Bank.[131]

Most recently, SAR have been published for dipeptide-based reactive methyl ketones containing 2-mercaptoimidazolium leaving groups (Fig. 4).[126] Their inhibitory potencies are strongly dependent on the substitution pattern of the imidazolium moieties. Substituents at the nitrogen atoms that are more bulky than a methyl group are not tolerated, but inhibitory potency was retained when the methyl substituents at C4 and C5 were replaced by propyl groups. Remarkably, these inhibitors lack selectivity between hTGase 2 and FXIIIa, whereas analogous dimethylsulfonium compounds are selective inactivators of hTGase 2. This result has

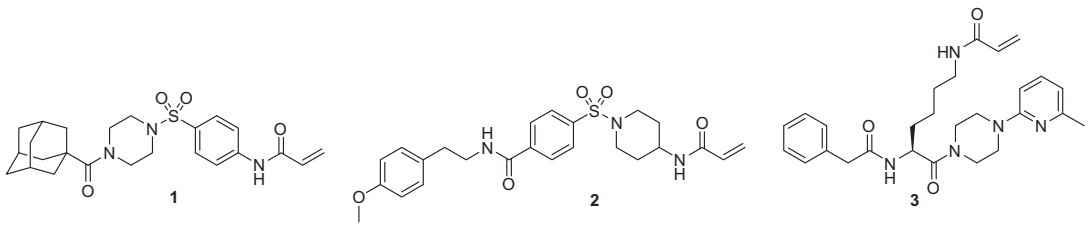


Scheme 6. TGase-catalyzed hydrolysis/aminolysis of the chromogenic substrate Cbz-E(ONp)G-OH and the fluorogenic substrates Cbz-G-γAbu-OCou and Cbz-G-γAbu-OCou. Product formation is quantified by measurement of *p*-nitrophenol absorbance ($\lambda = 400$ nm[118a,118b] or 410 nm[118c]) and fluorescence of 7-hydroxycoumarin ($\lambda_{ex} = 330$ nm, $\lambda_{em} = 460$ nm),[120a] respectively.



Scheme 7. TGase-catalyzed transamidation between the FRET system Abz-NE(Cad-Dnp)EQVSPLTLLK-OH (peptide sequence derived from α_2-antiplasmin) and H-G-OMe. The increase in fluorescence of the N-terminal 2-aminobenzoyl (Abz) due to release of the quenching 2,4-dinitrophenyl (Dnp) moiety is measured at λ_{ex} = 313 nm, λ_{em} = 418 nm.[123a]

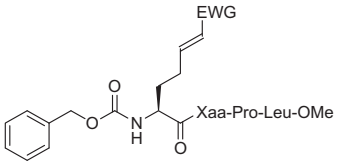
Table 4
Structures and inhibitory potencies of recently discovered acrylamide-based TGase 2 inhibitors



Compound	IC_{50} (nM)						Refs.
	hTGase 2	mTGase 2[a]	hTGase 1	hTGase 3	FXIIIa	hTGase 6	
1	10	16	3400	>80,000	180	840	102e
2	110	55	1240	Not tested	6400	34,000	102f
3	14	Not available	15,000	>80,000	35,000	>80,000	102g

[a] mTGase 2, murine TGase 2.

Table 5
General structure and inhibitory potencies of Michael-acceptor based peptidic hTGase 2 inhibitors disclosed in a patent application by Zedira GmbH[128]



Compound	EWG	Xaa	IC_{50} (nM)		
			hTGase 2	FXIIIa	hTGase 1
4	$CO_2CH_2CH_3$	Gln	20	>50,000	n.d.[a]
5	$CO_2CH_2CH_3$	Val	30	>50,000	n.d.[a]
6	SO_2CH_3	Gln	54	>100,000	250

[a] n.d., not determined.

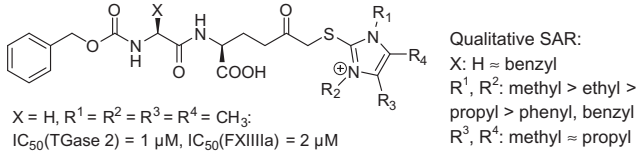


Figure 4. General structure of dipeptide-derived reactive methyl ketones containing a 2-mercaptoimdazolium leaving group and qualitative SAR for inhibition of hTGase 2 and FXIIIa.[126]

been explained on the basis of molecular modeling studies, which suggest that the imidazolium moiety interacts with Trp241 and Trp323, that is, residues that are present in both TGase 2 as well as FXIIIa. In the case of dimethylsulfonium inhibitors, the conformation around the reactive methylene group seems to differ from that in their imidazolium counterparts and favors cation-π interactions between the sulfonium group and Phe325 of TGase 2. This residue corresponds to Tyr372 in FXIIIa, which adopts a different conformation that does not promote cation-π interactions.[126]

Molecular imaging of TGase 2: There are two distinctive approaches to imaging TGase 2 in biological objects. The first category deals with the visualization of the enzyme on its own, based on the expression of recombinant TGase 2 fusion proteins that are covalently linked to fluorescent proteins. This approach was used to monitor the conformational transition between the closed and open form of the enzyme within its intracellular environment by fluorescence microscopy. For this purpose, hTGase 2 was conjugated with YFP and CFP at its N- and C-terminus, respectively.[132] The proximity of the termini in the closed conformation

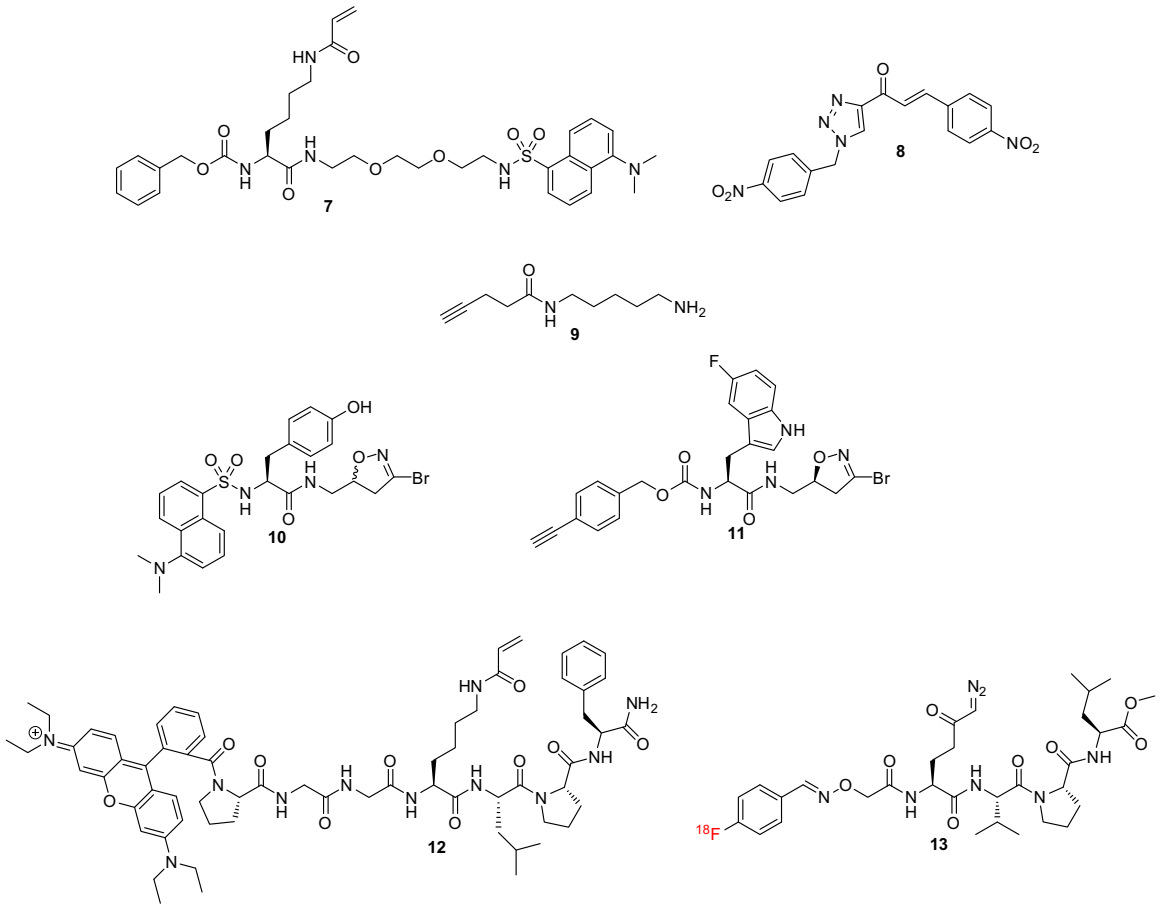


Figure 5. Inhibitors used to induce conformational changes within TGase 2 inside living cells (compounds **7** and **8**) and imaging probes to target TGase 2 in living objects (compounds **9**–**13**).

(distance 16 Å) results in FRET between CFP and YFP, which will be interrupted upon transition to the open conformation as the distance increases to 140 Å. Pavlyukov et al. expressed this construct in A549 and CHO cells as well as in mouse fibroblasts by transfection with the coding plasmid. Microscopic FRET monitoring in these cells revealed that a considerable amount of the enzyme is located in the perinuclear recycling compartment and is present in the form of closed, transamidase-inactive protein. Open, transamidase-active TGase 2 was detected close to the outer cell membrane. The transition of the conformation from close to open triggered by ionophore-mediated Ca^{2+} influx, and staurosporine-mediated apoptosis has been monitored using this FRET-based approach.[132a] In a similar study, Caron et al., revealed that the irreversible acrylamide-based inhibitor **7** (Fig. 5) induces the conformational transition to the open form in STHdh cells. In contrast, the cinnamoyl triazole **8** (Fig. 5) targeting the transamidase domain in a reversible manner stabilizes TGase 2 in the closed conformation.[132b]

Another approach to imaging TGase 2 relies on its targeting by exogenous agents functionalized with reporter groups. These agents can be based either on inhibitors or substrates. Regarding the latter class of imaging probes, cadaverine conjugated to fluorescein isothiocyanate (FITC) was used to detect transglutaminase activity in living cells.[133] Geel et al. visualized the TGase 2 activity in Movas smooth muscle cells by incubation with pent-4-ynoylcadaverine (Fig. 5, compound **9**) followed by fluorescent labeling with a commercially available Alexa555-azide probe via copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) and microscopic image analysis.[134]

The dansylated irreversible inhibitor **10** (Fig. 5) bearing a 3-bromodihydroisoxazole moiety was used to visualize TGase 2 in HCT-116 cells by two-photon confocal microscopy, revealing its presence on the surface and in minor quantities in cytosolic granules.[82a] Inhibitors of the same chemotype have been alkyne-functionalized (Fig. 5, compound **11**) to allow microscopic imaging of the TGase 2 activity in WI-38 fibroblast cultures.[82c] Fluorescence labeling after inhibitor targeting was achieved via CuAAC with azidopropylbiotinamide and incubation with streptavidin-conjugated Alexa555. This assay gave high signals with compound **11**, but no image signal was detected with the less potent epimeric analog of **11** exhibiting an inverted configuration of the dihydroisoxazole ring. The rhodamine-labeled peptidic acrylamide inhibitor **12** (Fig. 5) synthesized by Chabot et al. was used for microscopic detection of TGase 2 in aortic tissue slides from hypertensive rats treated with warfarin and vitamin K over four weeks. The fluorescence signal within the slides increased over time accompanied by relocation from the media to the adventitia, suggesting the enzyme's involvement in processes of fibrosis.[127e]

To the best of our knowledge, data on imaging and functional characterization of TGase 2 via single-photon emission computed tomography (SPECT) and positron emission tomography (PET) using radiolabeled probes in vivo are not available to date. However, the radiosynthesis of a fluorine-18 labeled peptidic diazomethyl ketone-based TGase 2 inhibitor **13** (Fig. 5) has been disclosed in a conference abstract.[135] Preliminary results from studies of our own institute toward ^{18}F-fluorbenzoylated diamines and spermine as substrate-based PET tracers have been published as conference abstracts and in annual proceedings.[136]

Summary: Within this digest Letter we introduced the reader to the physiological functions and pathological implications of TGase 2, emphasizing its role in tumor progression and biomaterial-tissue interactions. While results from recent research shed light into the enzyme's involvement in human disorders, some of its particular roles remain enigmatic, especially with regards to the different conformational and activity states of TGase 2. A survey of current methods to assay TGase transamidase activities was given. This will stimulate further medicinal chemistry efforts dealing with the development of inhibitors and imaging agents targeting TGase 2. Recent results in this field were also covered in the Letter.

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