INVITED REVIEW

Recent advances in the development of tissue transglutaminase (TG2) inhibitors

E. Badarau · R. J. Collighan · M. Griffin

Abstract Tissue transglutaminase (TG2) is a Ca^{2+}-dependent enzyme and probably the most ubiquitously expressed member of the mammalian transglutaminase family. TG2 plays a number of important roles in a variety of biological processes. Via its transamidating function, it is responsible for the cross-linking of proteins by forming isopeptide bonds between glutamine and lysine residues. Intracellularly, Ca^{2+} activation of the enzyme is normally tightly regulated by the binding of GTP. However, upregulated levels of TG2 are associated with many disease states like celiac sprue, certain types of cancer, fibrosis, cystic fibrosis, multiple sclerosis, Alzheimer's, Huntington's and Parkinson's disease. Selective inhibitors for TG2 both cell penetrating and non-cell penetrating would therefore serve as novel therapeutic tools for the treatment of these disease states. Moreover, they would provide useful tools to fully elucidate the cellular mechanisms TG2 is involved in and help comprehend how the enzyme is regulated at the cellular level. The current paper is intended to give an update on the recently discovered classes of TG2 inhibitors along with their structure–activity relationships. The biological properties of these derivatives, in terms of both activity and selectivity, will also be reported in order to translate their potential for future therapeutic developments.

Keywords Tissue transglutaminase · TG2 · Inhibitors · Structure–activity relationships (SAR)

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Abbreviations

TGase	Transglutaminase
SAR	Structure–activity relationship
HYD	Hydrophobic
HBA	Hydrogen bond acceptor
GTP	Guanosine triphosphate
GDH	Glutamate dehydrogenase
5-BP	5-Biotinamidopentylamine
tBoc	tert-Butyloxycarbonyl
Cbz	Benzyloxycarbonyl
Fmoc	Fluorenylmethyloxycarbonyl
Ac	Acetyl

Introduction

Transglutaminases are enzymes that perform post-translational modifications of proteins via an aminoacyltransferase reaction involving nucleophilic substitution at the gamma amide of a peptide-bound glutamine, with elimination of ammonia. The nucleophile may be the ε-amino group of a peptide lysine, another primary amine or water. In this way, $\varepsilon(\gamma$-glutamyl)lysine cross-links may be formed between proteins, peptide glutamines may be derivatised by primary amines, or peptide glutamines may be deamidated to glutamate. The protein cross-linking activity of transglutaminases is one of the most understood, resulting in the stabilisation of protein structures both against biological and mechanical degradation. Transglutaminases have been described in microorganisms, plants, invertebrates, amphibians, fish, birds and mammals. In mammals, transglutaminases are Ca^{2+} activated and important for the stabilisation of structures such as skin, hair and blood clots. The most ubiquitous mammalian transglutaminase is tissue

transglutaminase (TG2) and has been the subject of much research due to its association with a variety of disease states such as metastatic cancer, celiac disease, fibrosis and neurodegenerative disorders. The role of TG2 in the pathology of these diseases is still unclear, although transamidating activity has been shown to be important. The development of selective inhibitors that regulate TG2-transamidating activity therefore have the potential to act as novel therapeutic solutions. However, given the multi-functional roles of the eight active members of the mammalian transglutaminase family (Chen and Mehta 1999; Griffin et al. 2002), selectivity therefore concerns not only being selective for inhibition of transglutaminases but also being selective for inhibition of one or more of the transglutaminase members. Moreover, selectivity for particular transglutaminase members would help elucidate their specific biological roles.

To date, inhibitors of transamidation activity have been generally classified in two distinct subclasses depending on the inactivating mechanism of the enzyme: reversible (competitive or non-competitive) and irreversible inhibition. The competitive inhibitors are typically derivatives that include in their structure a medium to long saturated aliphatic chain bearing a terminal primary amine. This type of substrates were summarized previously (Lorand and Conrad 1984) and include among others putrescine and cadaverine-derived inhibitors, like 5-biotinamidopentylamine (5-BP), monodansyl cadaverine, fluorescein cadaverine (Fig. 1a–d). These type of compounds block cross-linking by acting as competitive primary amine substrates and become incorporated into theγ-glutamyl residue(s) of the peptide or protein substrate. Although useful for in vitro studies, in vivo they have limited

application since the aminated protein could feasibly elicit an autoimmune response. Moreover, they have the potential to act as competitive substrates for all the transglutaminase enzymes.

Many of the non-competitive reversible inhibitors reported up-to-date allosterically inactivate the enzyme by competing for the TG2 cofactor (GTP, GDP, etc.) binding site. Thieno[2,3-*d*]pyrimidin-4-one acylhydrizides identified by Case and co-workers (Duval et al. 2005; Case and Stein 2007) belong to this class of inhibitors (Fig. 1e). However, when designing such compounds, it must be taken into consideration that other members of the TG family, e.g. TG3 and TG5 also bind guanine nucleotides (Ahvazi et al. 2004; Candi et al. 2004).

Tissue transglutaminase irreversible inhibitors are probably the most widely studied type of TG2 inhibitors in the last 15 years. The majority target the active site CYS residue of the mammalian TGs, and incorporate in their structure an essential electrophilic warhead able to covalently react with the highly nucleophilic sulphur atom from the catalytic site CYS residue, therefore totally inactivating the enzyme. Importantly, using the information gathered from the active sites of crystal structures of TG3, Factor XIII and TG2 and from information gathered from the preferred peptide amino acid sequences acting as TGase substrates (Hitomi et al. 2009; Sugimura et al. 2011) they allow greater specificity to be achieved against the target TG.

The classical approach to design irreversible inhibitors was a "simplification" approach, which means designing derivatives structurally related to their preferred natural γ-glutamyl-containing substrates, but with a simplified chemical structure, more easy to modulate for the desired pharmacological profile. Several series of peptidic

Fig. 1 Chemical structures of some reversible TG2 inhibitors

(a) monodansyl cadaverine

(b) biotinamidopentylamine

(c) putrescine

(d) fluorescein cadaverine

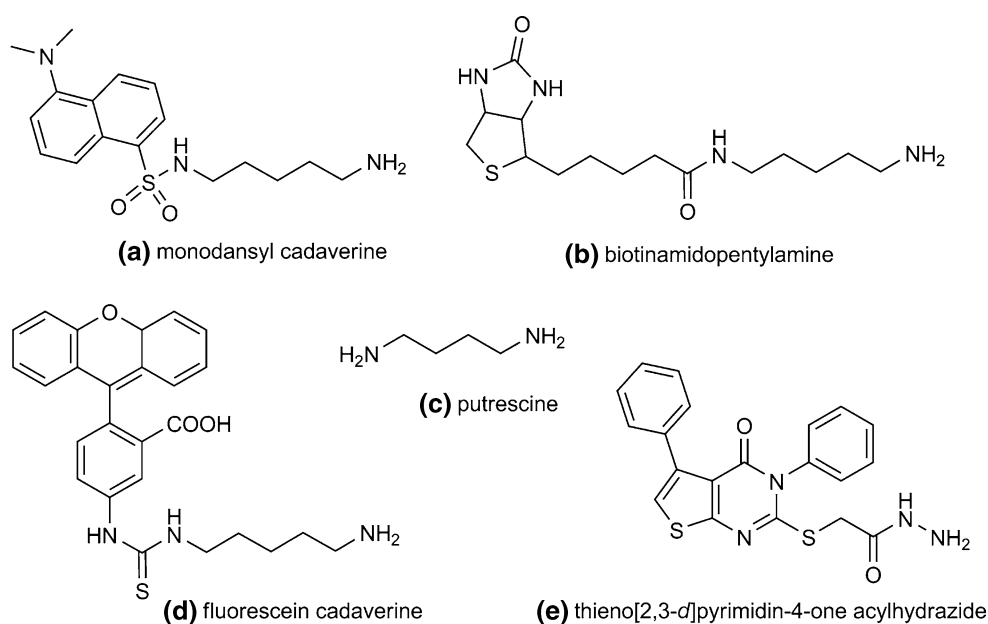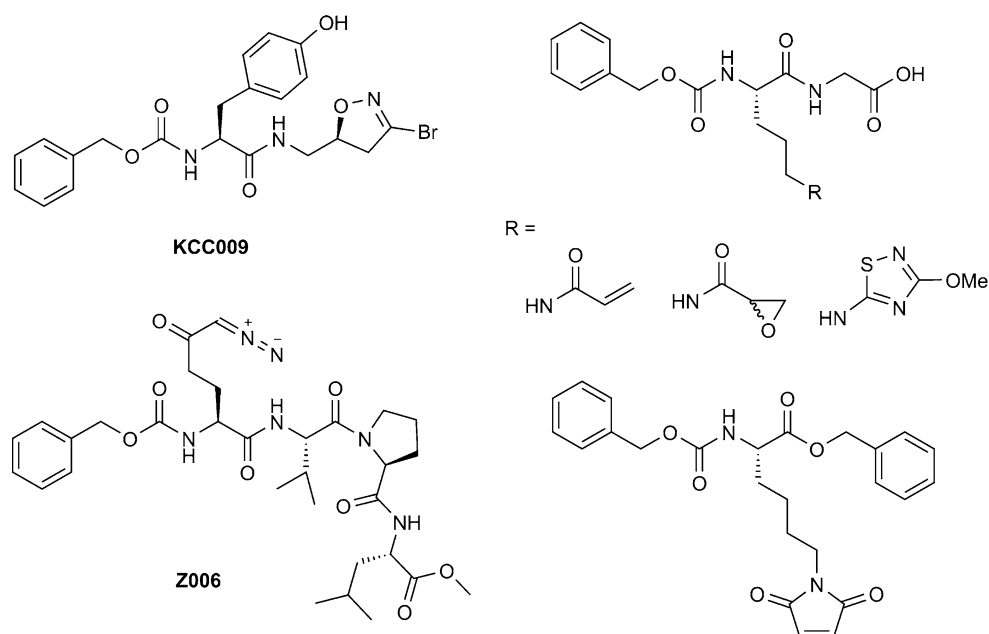(e) thieno[2,3-*d*]pyrimidin-4-one acylhydrazide

Fig. 2 Examples of previously reported peptidic irreversible inhibitors

inhibitors emerged following this concept, the chemical structure of the warhead being generally used to differentiate among them (Fig. 2): 3-halo-4,5-dihydroisoxazoles (Choi et al. 2005; Watts et al. 2006; Castelhano et al. 1988), epoxides (de Macedo et al. 2002; Marrano et al. 2001b), α,β-unsaturated amides (de Macedo et al. 2002; Marrano et al. 2001b; Pardin et al. 2006), chloroacetamides (Pardin et al. 2006), thiadiazoles (Marrano et al. 2001a), maleimides (Halim et al. 2007; Pardin et al. 2006), sulfonium methyl ketones (Pliura et al. 1992).

One of the most promising inhibitors described to date belongs to the dihydroisoxazole family (KCC009, Fig. 2). With a good pharmacokinetic profile and a good bioavailability in mice, this compound was tested in a human colon cancer cell line where it was shown to increase chemo-sensitivity of the tumour (Choi et al. 2005) in the range 1–100 μM. However, Verhaar et al. (2011) showed that KCC009 was unable to affect TG2 activity up to a concentration of 100 μM in their SH-SY5Y cell lines. Contrarily, the 6-diazo-5-oxo-noerleucine (DON) derivative Z006 which penetrates into cells (Fig. 2) induced a near complete inhibition of cellular TG2 at a concentration of 10 μM (20-fold higher than in in vitro assays). Moreover, KCC009 does not affect the TG2-regulated formation of Mallory Bodies inside hepatocytes (Strnad et al. 2006) suggesting it may have limited intracellular activity.

Recently discovered classes of tranglutaminase 2 inhibitors

The main intention of this article is to review the recently reported TG2 inhibitors along with their structure–activity

relationships (SAR) and their known specificity and applications to date. For a comprehensive view on this topic, the reader is also suggested to consult the previously published reviews (Siegel and Khosla 2007; Wodzinska 2005).

Cinnamoyl derivatives as TG2 inhibitors

Starting from a comparative study between Cbz-Gln-Gly versus Boc-Gln-Gly-binding modes via molecular modeling (Chica et al. 2004) but also from the privileged interaction of the benzyloxycarbonyl (Cbz) moiety from several TG2 peptidic inhibitors with the enzyme's binding site, the group of Keillor further investigated the importance of rigidity of this carbamate moiety (Pardin et al. 2008a). Their efforts led to the identification of *trans*-cinnamoyl derivatives as competitive reversible TG2 inhibitors (Fig. 3), with IC_{50} values for guinea pig liver TGase ranging from 18–>200 μM. The most potent inhibitors were divided in two subclasses, depending on the nature of the heteroaromatic substituent (HET) moiety: benzotriazolyl or pyridinyl.

Initial SAR on the aromatic cinnamoyl scaffold showed that substituents in the para position (preferably with a sp_2 hybridized oxygen, e.g. –NO$_2$, BocHN–, FmocNH–, AcNH–, –COOMe) positively influenced the affinity, while other substituents (Me–, MeO–, Cl–) had an unfavourable effect on binding. The observed IC_{50} values were as low as 18 μM in the case of *p*-BocHN substituent and a 3-pyridyl as the HET moiety. The biological evaluation of their flexible analogues (the alkene is reduced or replaced by methylene-ether) showed a loss of activity. Moreover, the introduction of a supplementary conjugated alkene was

Fig. 3 General structure of
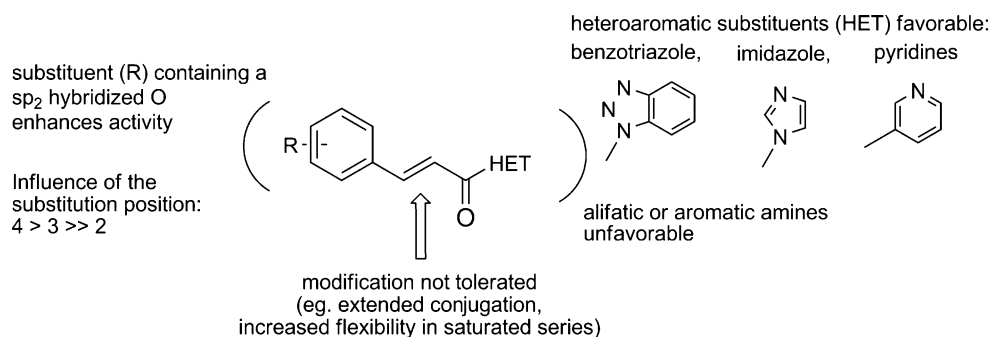cinnamoyl derivatives and their
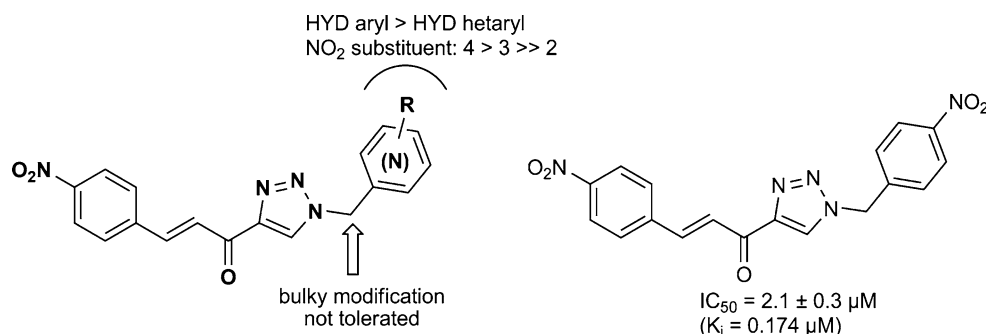SAR tendencies (Pardin et al.
2008a)

substituent (R) containing a
sp$_2$ hybridized O
enhances activity

Influence of the
substitution position:
4 > 3 >> 2

heteroaromatic substituents (HET) favorable:
benzotriazole,   imidazole,   pyridines

alifatic or aromatic amines
unfavorable

modification not tolerated
(eg. extended conjugation,
increased flexibility in saturated series)

Fig. 4 General structure of
triazole transglutaminase
inhibitors and their SAR trends
(Pardin et al. 2008b)

HYD aryl > HYD hetaryl
NO$_2$ substituent: 4 > 3 >> 2

bulky modification
not tolerated

IC$_{50}$ = 2.1 ± 0.3 μM
(K$_i$ = 0.174 μM)

detrimental to the TG2 affinity, compared to their shorter
analogues. Screening of several amines (aliphatic or aro-
matic) as the HET moiety had a negative impact on the
affinity, as well as Bz and *t*BuO ethers. The overall con-
ducted modulations showed that an aromatic moiety pos-
sessing hydrogen bond acceptors (HBA) is mandatory for a
good affinity in this series of compounds (Fig. 3). This
group of inhibitors seem to have a good selectivity profile
since no inhibition of FXIII or caspase-3 was detected up to
200 μM as showed by direct continuous colorimetric
assays (Leblanc et al. 2001).

Further development of this cinnamoyl series was
reported subsequently by the same group (Pardin et al.
2008b). Starting from the hypothesis that an aromatic
heterocycle containing HBA atoms was essential for a
good affinity (see above), various substituted triazoles were
synthesized and evaluated (Fig. 4).

The conducted modulations showed that an alkyl sub-
stituent on the triazole ring generally induced a onefold
increase of affinity. For example, compared to the parent
non-substituted triazole (IC$_{50}$ = 45 ± 0.5 μM), introduc-
ing a benzyl substituent resulted in an IC$_{50}$ decrease to
4.3 ± 0.3 μM. The interaction between the triazole sub-
stituent and the binding site is most probably hydrophobic,
since a cyclohexylmethyl substituent retains the affinity
(although slightly lower) compared to its aromatic ana-
logue (IC$_{50}$ = 11.0 ± 1.5 μM vs. IC$_{50}$ = 4.3 ± 0.3 μM).
The newly discovered inhibitor (Fig. 4) did not protect or
potentiate cell death induced by oxygen–glucose depriva-
tion when used on cells expressing wild type TG2. Up to its

toxicity limit (20 μM) it did inhibit ionomycin-induced
increases of transamidation activity for cells expressing the
wt TG2 and in cells transfected with the TG2-R580A
mutant (Colak et al. 2011).

Although the reversible cinnamoyl-based inhibitors
were in silico modelled to accommodate the acyl donor
substrate-binding site, further empirical proof was neces-
sary to sustain this hypothesis. A new derivative (Fig. 5)
incorporating a diazirine warhead was designed and
enabled photolabelling of TG2 residues in its proximity, in
the active-binding pocket (Pardin et al. 2009). Due to the
ability of alkyl diazirines to generate diazo and carbene
derivatives by photoactivation (Brunner 1993; Ziebell et al.
2004), this photolabile inhibitor was proved by mass
spectrometry to covalently react with the Cys230 residue.
Native polyacrylamide gel electrophoresis showed that the
enzyme-bound inhibitor adopts an open-form conformation
[analogous to the reported crystal structure resolved in
2007 (Pinkas et al. 2007)], despite contradictory molecular
modelling studies by the same authors, which suggested a
more solvent exposed Cys230 residue in the closed
conformation.

Inhibitors identified by the screening of libraries

Several potent hits for TGase were identified during high-
throughput screening assays (Lai et al. 2008) of two
libraries of pharmacologically active compounds: LOPAC
(1,280 cpds) and Preswick (880 cpds). The most interest-
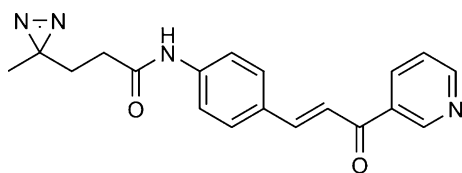ing derivatives validated via a first soluble-phase

Fig. 5 Structure of the cinnamoyl photolabile inhibitor (Pardin et al. 2009)

fluorescent dansylated lysine (KxD) incorporation assay and a subsequent colorimetric biotinylated pentylamine (BP) incorporation assay (which eliminate fluorescence interference) were subjected to a Triton X-100 test, known to eliminate false-positives induced by non-specific small-molecule aggregates (McGovern et al. 2003). Figure 6 summarizes the most interesting hits found following this approach.

All the compounds inhibited TG2 irreversibly in the presence of Ca^{2+}. However, when reducing agent DTT was included in the transglutaminase reaction their activity was significantly reduced, which the authors suggested was due to the inhibitors promoting the formation of disulfide bonds that inactivated the enzyme. Surprisingly, in the absence of Ca^{2+} several ligands were found to reversibly bind to the enzyme but Tyrphostin-47 was the only inhibitor, which competed with the GTP for its binding to TG2. Concerning the selectivity profile, beside their original protein targets listed in the screening database, the compounds were tested against blood coagulation factor FXIII, by using the same biotin pentylamine incorporation assay. They were found to be equipotent for FXII as for TG2, except for the vitamin K3, which had a sixfold decrease of affinity for FXIII.

A more complete biological profile for these hits, but also for other several classes of transglutaminase inhibitors, was reported by Macdonald and co-workers (Schaertl et al.

2010). During their efforts for developing a profiling platform for characterizing TG2 inhibitors, the authors investigated the TGase activity over a panel of transglutaminase isoforms (TG1, TG2, TG3, TG6 and FXIII), but also conducted a number cell activity studies and toxicity assays. However, it should be mentioned that, in the case of cellular assays, no data indicating the membrane permeability were reported. The biological results for the hits previously discovered by screening (Lai et al. 2008) are presented in Table 1.

Beside the biological characterization of previously reported inhibitors, the authors also revealed the SAR for new quinazolinone derivatives. Developed from the acylhydrazides published by Cuny and co-workers (Duval et al. 2005), their efforts led to the discovery of a highly potent (20 nM) and selective inhibitor for TG1, TG3 and FXIII. However, no specific structures and additional cellular data were disclosed. The overall SAR trends in this series are presented below (Fig. 7).

β-Aminoethyl ketones as TG2 inhibitors

Even if the IC_{50} of the previously reported hit ZM 39923 [$IC_{50} = 0.01 \pm 0.01$ μM (Lai et al. 2008); Fig. 6] was found to be only 4 μM in their activity assays, this derivative was the starting point of an extensive SAR study published by Mikoshiba and co-workers (Ozaki et al. 2010). The authors stated that the original β-aminoethyl ketone framework proved essential for the TGase binding, contrary to their α- or μ-aminoalkyl ketone analogues. Various aromatic ketones in both carbocyclic and heterocyclic series were investigated: heteroaromatic ketones appeared to be more favoured compared to the aromatic substituents, thiophene and furan showing the best results. The most potent ligand from this series as well as the SAR

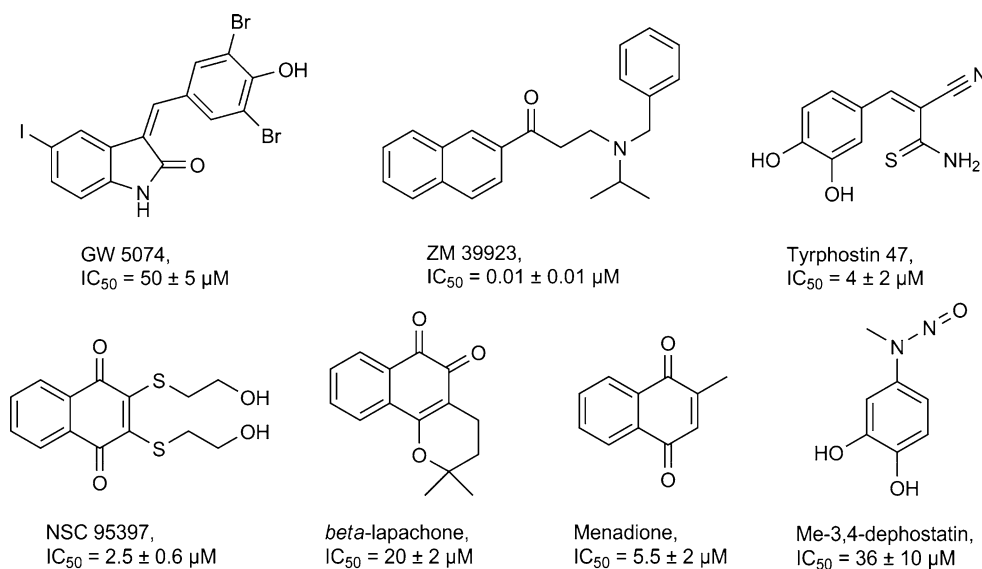Fig. 6 Hits discovered via a HTS approach (Lai et al. 2008)

GW 5074,
$IC_{50} = 50 \pm 5$ μM

ZM 39923,
$IC_{50} = 0.01 \pm 0.01$ μM

Tyrphostin 47,
$IC_{50} = 4 \pm 2$ μM

NSC 95397,
$IC_{50} = 2.5 \pm 0.6$ μM

beta-lapachone,
$IC_{50} = 20 \pm 2$ μM

Menadione,
$IC_{50} = 5.5 \pm 2$ μM

Me-3,4-dephostatin,
$IC_{50} = 36 \pm 10$ μM

Table 1 IC$_{50}$ (µM) values obtained for the screening hits (see Fig. 6 for molecular structures), modified from (Schaertl et al. 2010)

Compound	hTG2	mTG2	Cellular TG2	hTG1	hTG3	FXIIIa	Caspase-3	Tox
GW 5074	4.6	10.6	1	5.7	2.7	3.9	70	25
ZM 39923	2.8	1	>50	2.9	>80	1.7	47	50
Tyrphostin 47	45	59	>50	16	6.4	11.4	>50	0
NSC 95397	1.3	2.1	>50	0.93	0.5	0.41	>50	30
β-Lapachone	0.75	1.1	>50	0.6	0.38	0.37	0.19	3
Menadione	2.6	6.6	>50	2.9	1.8	2.1	6	20

Fig. 7 SAR trends of acylhydrazides (Duval et al. 2005; Schaertl et al. 2010)
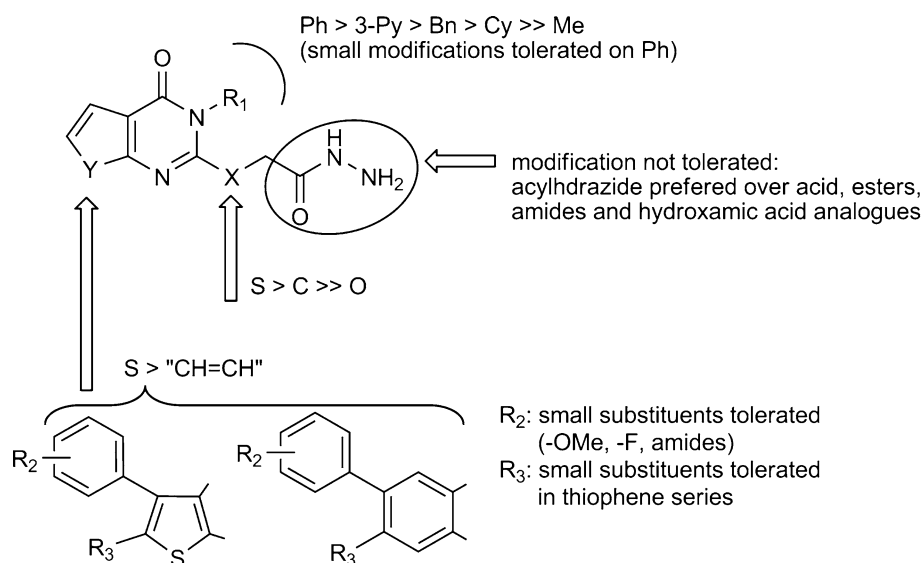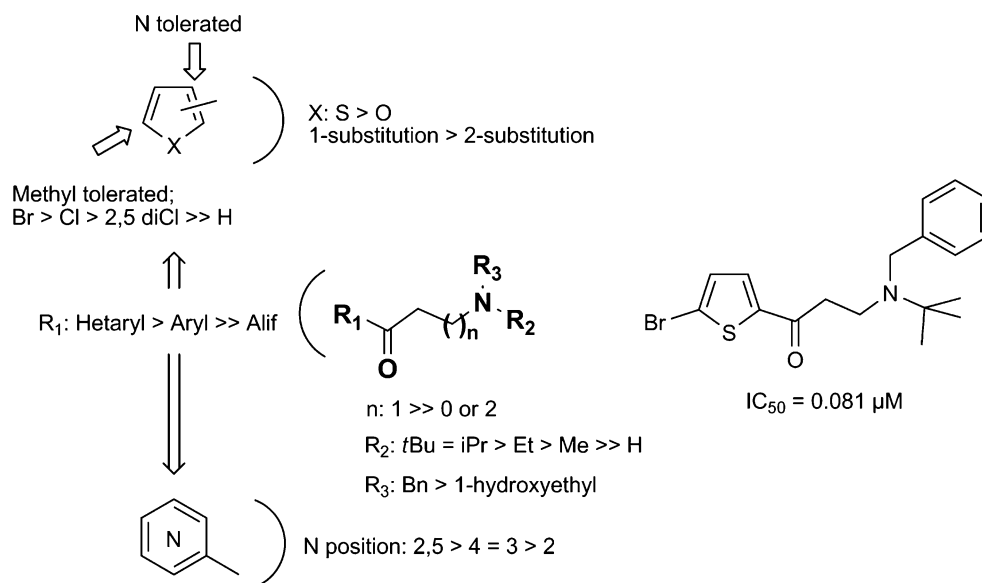

Fig. 8 SAR trends and the most potent derivative in β-aminoethyl ketone series (Ozaki et al. 2010)

trends from the investigated compounds are presented below (Fig. 8).

In relation to the well-known affinity of disulfide compounds for transglutaminases (Connellan and Folk 1969; Chung and Folk 1970), of which recent examples include cystamines (Okauchi et al. 2009), the group of Mikoshiba

has developed a series of disulfide dimers starting from the same β-aminoethyl ketone building block (Ozaki et al. 2011). This new series of derivatives, when tested for their effects on TGase activity (Ozaki et al. 2010), showed affinities in the 0.12–19 µM range. Again, the thienyl ketone substituent conserved the best interaction with the

binding site of TG among all the tested aromatic substituents (Fig. 9).

Oxindole-based TG2 inhibitors

The screening of the Navigator's iResearch library for TG2 inhibitors by Griffin and co-workers, led to the identification of various 2-oxindoles derivatives with TGase inhibitory activity (Klock et al. 2011). Besides some dimers with μM affinity for TG2, the screening also identified a more interesting compound, prone to further pharmacomodulations: 2-acylidenoxindole (Fig. 10). It is worth mentioning that the same scaffold was discovered earlier (Lai et al. 2008) during their screening assays (compound GW 5074, Fig. 4). Modulation of substituents R_1, R_2 and R_3 (Fig. 10) showed that the oxindole's aromatic moiety is the most
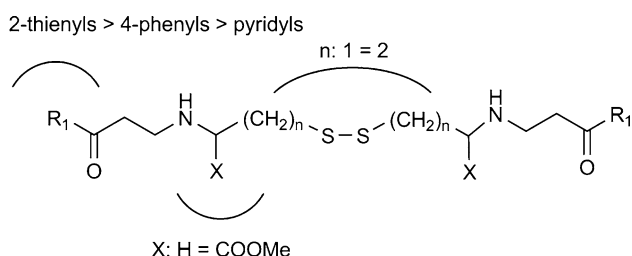

Fig. 9 SAR tendencies for the dithio-β-aminoethylketones (Ozaki et al. 2011)

sensitive region of the molecule for the interaction with the TG2-binding site, a –Br or –Cl substituent on C_4 enhancing the activity for at least sixfold compared to their unsubstituted analogues.

As underlined in Fig. 10, the compounds were submitted to two different binding assays, a GDH-coupled assay and a more sensitive coumarin-based fluorescence assay, which allows the use of the TG2 enzyme at concentrations below 100 nM. Interestingly, this fluorometric assay showed that several compounds from this series acted either as partial inhibitors or non-competitive, allosteric inhibitors.

Recently discovered peptidic TG2 inhibitors

In an extension to their previous studies on the peptidic dihydroisoxazoles (Watts et al. 2006) as TG2 inhibitors, Dafik and Khosla (2011) recently reported new derivatives with interesting TGase inhibitory activity. Developed for labelling and visualizing the catalytically active form of TG2, these compounds incorporate reactive azido or alkynyl functional groups designed for trapping fluorophores or different tags by using alkyne-azide "click" chemistry, as illustrated previously in the literature (Sletten and Bertozzi 2009). The conducted modulations also included the aminoacid moiety in Trp, Tyr or Pro series, the overall SAR trends, observed using kinetic assays on

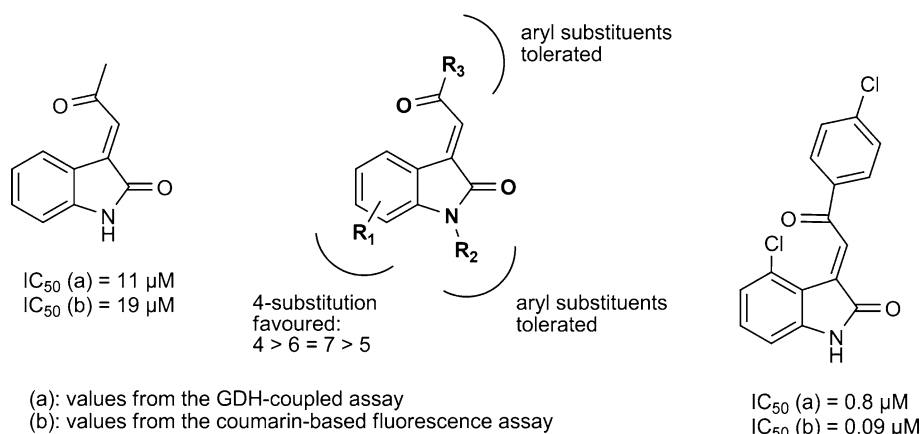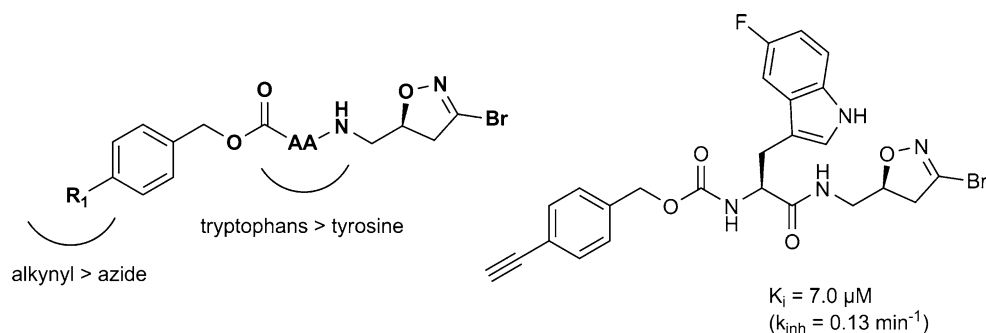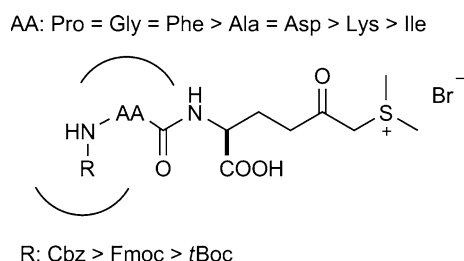Fig. 10 Initial hit from screening, SAR trends and the most potent compound in oxindole series (Klock et al. 2011)

Fig. 11 SAR trends and one example of inhibitor from the recently reported peptidic dihydroisoxazoles (Dafik and Khosla 2011)

AA: Pro = Gly = Phe > Ala = Asp > Lys > Ile

R: Cbz > Fmoc > tBoc

Fig. 12 General structure and SAR of water-soluble TG2 inhibitors (Griffin et al. 2008)

hTG2, are presented bellow (Fig. 11). Some of the synthesized compounds were also tested in a tissue culture wounding model, using the concept validated earlier (Siegel et al. 2008), the most potent inhibitors completely blocking TG2 activity around the "wound" at concentrations as low as 3.1–6.25 μM.

In their search for aqueous soluble TGase inhibitors that could target preferentially the extracellular pool of TG2, since many of the pathologies that the enzyme is involved in such as fibrosis, celiac disease, multiple sclerosis and cancer are either wholly or partially limited to the extracellular space, Griffin et al. (2008) developed a series of sulfonium peptidylmethylketones related to those published by Pliura et al. (1992). The carboxylic acid and the dimethylsulfonium moieties are key distinguishing features that increase the compound's solubility in water, due to the presence of charges at physiological pH. Beside the variation of the central aminoacid core, the authors also investigated the modification of the carbamate moiety (Fig. 12). Several other warheads based on the imidazolium scaffold could also be tolerated or even enhance the TGase activity (Griffin et al. 2004). Members of this series of compounds have been used successfully in cell studies (Baumgartner et al. 2004; Jones et al. 2006; Telci et al. 2009) and in preclinical studies of diabetic nephropathy and kidney scarring where they induced no animal toxicity up to 120 days and successfully reduce kidney fibrosis and scarring by up to 85% with a significant increase in kidney function (Johnson et al. 2007, 2008; Huang et al. 2009)

Concluding remarks

The increasing discovery of tissue transglutaminase involvement in many physiological processes and disease states make it an attractive therapeutic target. Publications in this area are constantly increasing, with the last 4 years being extremely rich in new classes of discovered inhibitors. The usefulness of the screening approaches used for the identification of new active compounds has been confirmed by a number of groups. The reported crystal structures for three of the TG family have further aided inhibitor

design and in the case of TG2 revealed the complex conformational dynamics of this multifunctional enzyme. This has led to a more rational drug design phase which no doubt will expand in the immediate future leading to further improvements in the potency and selectivity of possible drug candidates. However, the discovery of a first drug that alleviates one of the disease states that TG2 is involved in by modulation of enzyme function remains a challenging task for the research community.

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