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Short communication

Imidazolium-based warheads strongly influence activity of water-soluble peptidic transglutaminase inhibitors

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ABSTRACT

New peptidic water-soluble inhibitors are reported. In addition to the carboxylate moiety, a new polar warhead was explored. Depending on the size of its substituents, the newly appended imidazolium scaffold designed to enhance the hydrophilic character of the inhibitors could induce a good inhibition for tissue transglutaminase (TG2) and blood coagulation factor XIIIa (FXIIIa). Correlated with the narrow tunnel that hosts the target catalytic cysteine residue, the various modulations suggest a bent conformation of the ligands as the binding pattern mode. Analogues in the dialkylsulfonium series were also tested and showed specificity for TG2 over FXIIIa.

1. Introduction

Transglutaminases (TGs) are a group of enzymes that catalyse the formation of protein crosslinks by forming isopeptide bonds between peptide bound glutamine and lysine residues, either in an inter- or intramolecular manner [1]. Although under normal physiological conditions they are responsible for the stabilisation of high molecular weight protein structures e.g. in skin and in hair, abnormal levels of transglutaminases in particular the ubiquitous TG2 are associated with many disease states. These include neurodegenerative diseases, celiac sprue, cataract and fibrosis [2].

Starting from the hypothesis that in many disease states such as fibrosis, celiac sprue and cancer, transglutaminases such as TG2 act extracellularly, we initiated a medicinal chemistry programme aimed at developing tools for such diseases. The design of highly hydrophilic inhibitors that could control the over-expressed levels of extracellular TG2 associated with the above-mentioned pathologies would also have the advantage of a desirable cellular toxicity profile for such molecules, as their intracellular access would be restrained. These were, in fact, the fundamentals when we developed a new series of TG2 inhibitors incorporating, in addition to the polar dimethylsulfonium warhead pioneered by Pliaura et al. in early 90s [3], a new carboxylate moiety [4]. As a proof of this concept, such polar molecules were successfully used in preclinical studies of diabetic nephropathy and kidney scarring where they induced no animal toxicity up to 120 days and successfully reduced kidney fibrosis and scarring by up to 85% with a significant increase in kidney function [5–7].

As a continuation of our research programme on TG2, we synthesized new analogues by replacing the previous warhead (dimethylsulfonium) with a new polar moiety, namely imidazolium-based warheads. The discovery that imidazolium derivatives inhi bit transglutaminases was previously disclosed by Merck in 1992 [8] (A, Fig. 1). Subsequently, the same group showed that the key structural feature for the irreversible reaction with the enzyme is in fact the acetonyl-thioimidazolium. Acetonylation of the catalytic CYS residue would thus render the enzyme inactive while releasing the imidazolium thione [9].

Since such small compounds patented by Merck have many drawbacks for future pharmacological use in terms of TG specificity and target attainability, we incorporated this imidazole feature into our previously developed inhibitors. The hydrophilic character of our new compounds would be preserved due to the presence of both carboxylate and imidazolium salts in their structure (Fig. 1). As the imidazolium-based derivatives were patented by Merck for their ability to inhibit the FXIIIa, we evaluated our new series on both TG2 and FXIIIa.

Several crystal structures for TG2 have been published. They revealed a huge conformational change for this enzyme between the nucleotide-bound conformation [11,12] ("closed"

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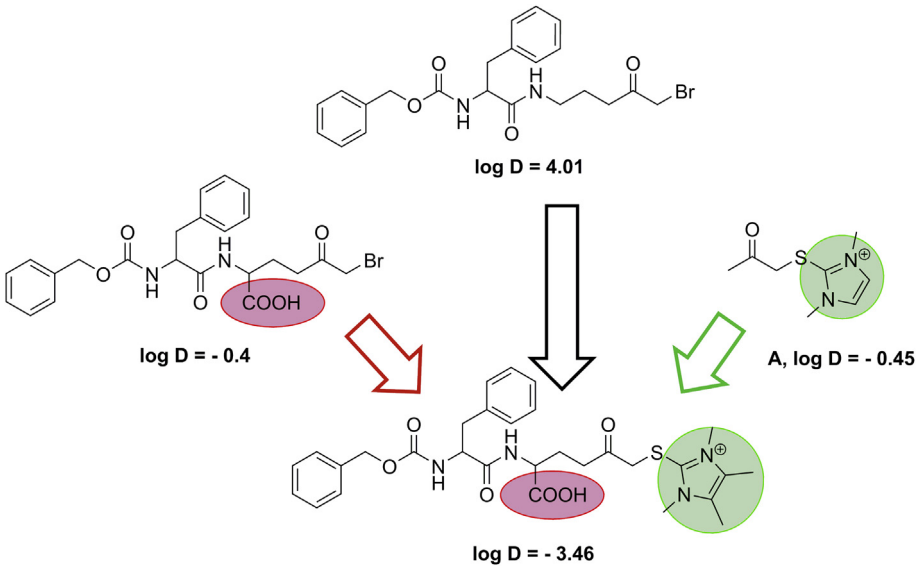


Fig. 1. Influence of polar groups on the hydrophilicity of newly-designed compounds. Log *D* values at pH = 7.4 in an octanol/water mixture were predicted using SPARC Software [10].

conformation) and an irreversible inhibitor-bound conformation [13,14] ("open" conformation). As the key catalytic CYS residue was revealed to be hosted in a narrow tunnel, several research groups assumed that the electrophilic warhead should be size-limited, in order to reach and react irreversibly with the nucleophilic CYS residue. Examples of such small-size warheads are α,β-unsaturated amides [15–17], chloroacetamides [17], epoxides and vinyl acetamides [15,16] and maleimides [17,18]. We were thus intrigued to find out if appending a bulky imidazolium moiety to our previous peptidic inhibitors would interfere with the binding and/or bonding with the enzyme. Although the reaction with the CYS277 residue is mechanistically different than in the case of thioimidazolium warheads, we were encouraged by the finding that other bulky group such as thiadiazoles could also be accommodated by the binding site [19].

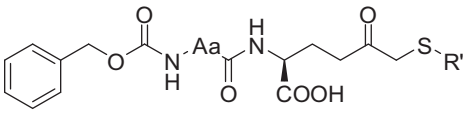
2. Results and discussion

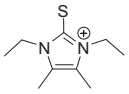
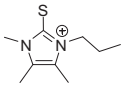
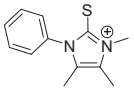
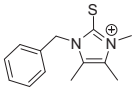
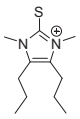
Our studies started with the synthesis of the imidazolium-based derivative **1a** (Table 1). This compound inhibited TG2 with an IC_{50} of 3 ± 1 μM, comparable with the most potent derivatives in the dimethylsulfonium series [4]. When tested in Swiss 3T3 cell lines, this inhibitor did not show any toxicity up to 250 μM for 72 h, indirectly confirming that it would successfully target only the extracellular TG2. Encouraged by these preliminary results, we subsequently decorated the imidazolium scaffold with various substituents in order to evaluate the influence of these structural modifications on TG2 inhibition.

2.1. Chemistry

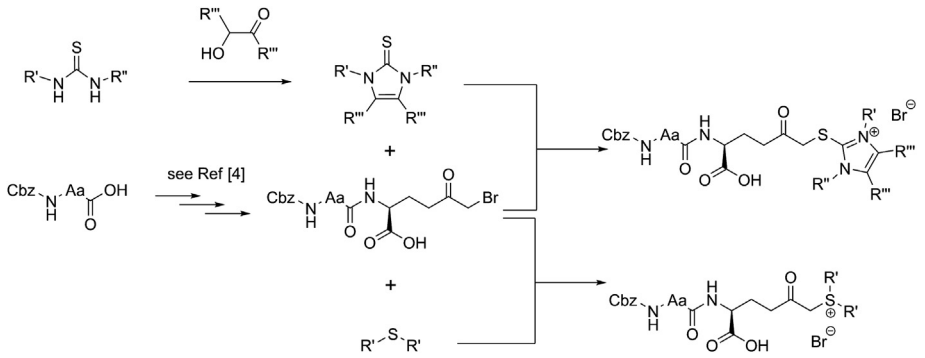
As the first step in the synthesis, a set of imidazolium-thiones was obtained by condensation of different thioureas and acetoins in refluxing hexan-1-ol. Because the catalytic CYS277 residue was revealed by crystallography to be situated in a small cavity (PDB codes: 1KV3, 2Q3Z), different sizes and positions of the substituents (Me, Et, Pr, Ph, Bn) on the imidazolium moiety were tested. The obtained thiones were reacted with the bromo-methylketones synthesized as previously described by our group [4] (Scheme 1). All modifications were conducted in both the glycine and the phenylalanine series. We were interested in testing if these different amino acids as the central substituents (H for GLY vs Bn for PHE) could induce different conformational change for the final derivatives in their binding to the enzymes and whether they had any influence on the potency of the compound for TG2 compared with FXIIIa.

Table 1
Inhibition results in case of imidazolium-based derivatives.



S-R′	Aa = PHE			Aa = GLY		
		IC_{50} (TG2)	IC_{50} (FXIII)		IC_{50} (TG2)	IC_{50} (FXIII)
	**1a**	3	5	**1b**	1	2
	**2a**	>200	50	**2b**	>200	>200
	**3a**	>200	>200	**3b**	>200	30
	**4a**	>200	>200	**4b**	>200	>200
	**5a**	>200	>200	**5b**	>200	>200
	**6a**	5	10	**6b**	10	20

IC_{50} values are reported in μM and represent the average of 2–3 different experiments run in triplicate for which SD ≤20%.



Scheme 1. Synthesis of target inhibitors.

In order to correlate better the new imidazole-based derivatives with our previous inhibitors, analogues in the dialkylsulfonium series were also synthesized and biologically tested. The alkyl substituents on sulfonium warhead were varied in methyl and ethyl groups. The newly synthesized derivatives were characterised by infra-red spectroscopy (IR), high resolution mass spectrometry (HRMS), proton and carbon-13 NMR spectroscopy (^{1}H and ^{13}C NMR), melting points, and had properties consistent with the proposed structures (see Supporting Information).

2.2. Inhibition activity

The final molecules were evaluated for their inhibitory activities for TG2 and FXIIIa (Tables 1 and 2). Briefly, as previously described, the enzyme assay in which the inhibitors were evaluated for their IC_{50} was based on the Ca^{2+}-mediated incorporation of *N*-(5-aminopentyl)biotinamide into *N*,*N*′-dimethylcasein by recombinant human TG2 as previously described by Griffin et al. [4] (see Supporting Information).

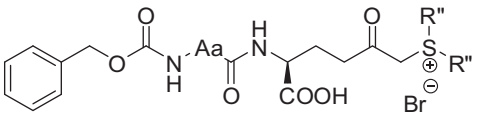
The first important remark concerning the IC_{50} results for TG2 is that the overall inhibition trends are the same in the PHE series (**1a**–**6a**) compared to the GLY series (**1b**–**6b**). Thus, despite the increased conformational freedom of GLY-based molecules, as there is practically no discrimination between the activities of the compounds with a GLY versus PHE core, we can conclude that the binding pattern in both series is most probably the same.

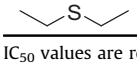
Concerning the conducted structural modifications (Table 1), one can observe that the activity is extremely sensitive to modifications of the substituents on imidazolium nitrogens. In fact, when passing from dimethyl to diethyl substituents (**1a**,**b** vs **2a**,**b**) the inhibition activity is lost. The same dramatic decrease of activity is observed even when only one nitrogen substituent is increased in size (**1a**,**b** vs **3a**,**b**, **4a**,**b** and **5a**,**b**). The fact that the activity is gradually lost with the increase of the size of the substituent was confirmed for a monoethyl substituent (data not presented). The hypothesis that extra aromatic substituents like phenyl or benzyl (**3a**,**b** or **4a**,**b**) could perhaps stabilise the initial ligand binding by interaction with aromatic residues around the catalytic CYS277 residues (e.g. TRP241, TRP323 or PHE325) proved unsuccessful. Interestingly, modifications of substituents on C4 and C5 positions of imidazolium scaffold were tolerated, as compounds **6a**,**b** retained a good inhibition range.

In the alkylsulfonium series, a similar activity trend was observed. Small substituents favoured a better binding profile, whilst increasing their size induced a fall of activity (**7a**,**b** vs **8a**,**b**).

Concerning the specificity profile of these water-soluble inhibitors, it was discovered that the dialkylsulfonium warheads have privileged interactions with TG2 residues, while these are not present in the case of FXIIIa. The imidazolium warheads could not discriminate between the two extracellular transglutaminases, even if a certain trend could be identified in the case of **2a** and **3b**. Thus, this type of compound is either equipotent for both TG2 and FXIIIa (**1a**,**b** and **6a**,**b**) or devoid of any inhibition trend (**2a**,**b**–**5a**,**b**).

Table 2
Inhibition results for dialkylsulfonium derivatives.



R″–S–R″	Aa = PHE			Aa = GLY		
		IC_{50} (TG2)	IC_{50} (FXIII)		IC_{50} (TG2)	IC_{50} (FXIII)
	7a	10	105	**7b**	8	>200
	**8a**	20	>200	**8b**	20	>200

IC_{50} values are reported in μM and represent the average of 2–3 different experiments run in triplicate for which SD ≤20%.

2.3. Molecular modelling

The fact that small structural modifications induce a huge activity change for TG2 (**1a**,**b** vs **2a**,**b**) seems to be in accordance with the fact that the catalytic CYS residue is situated in a narrow cavity which has a very minor conformational change in both steps of the inhibition process: the formation of the reversible Michaelis–Menten complex and the subsequent covalent bonding of the inhibitor. In fact, when the closed and the extended conformations of TG2 are superimposed in the core region (PDB codes: 1KV3 vs 2Q3Z), little change is observed around the key CYS277 residue. The inhibitors should thus have a strong complementarity with the enzyme in the warhead region, as the dynamics of the induced fit process is limited in CYS277 area, but more extensive in the adjacent hydrophobic and loop regions.

Preliminary docking studies showed that, in order to form viable complexes with the enzyme, the inhibitors from the newly synthesized imidazolium family should adopt a bent conformation. As the irreversibility of the reaction was verified in the case of our potent inhibitors by dilution experiments (as previously described [4]), a distance constraint between the electrophilic carbon of the inhibitor and the nucleophilic sulphur atom of the CYS277 residue was imposed during the docking process as implemented in the Gold software [20]. Compound **6a** was selected as reference for our

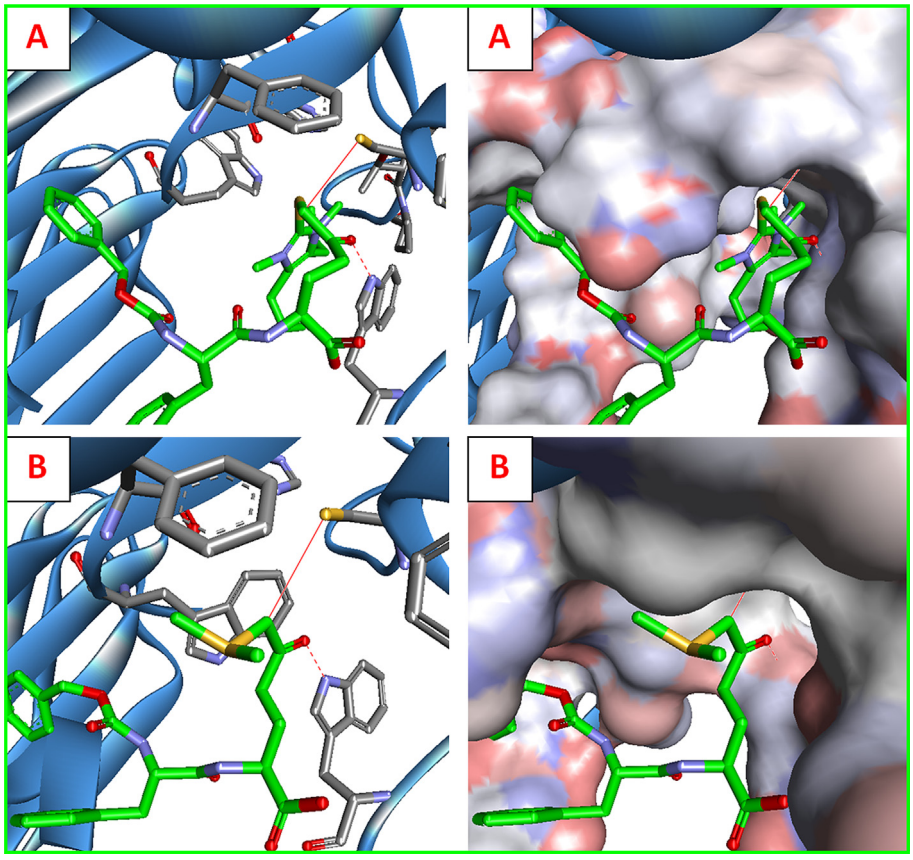


Fig. 2. Selected TG2 complexes with **6a** (A) and **7a** (B) derived from docking. The solvent accessible surface around the ligand is also represented (right). The continuous red line represents the distance between the electrophilic carbon of the ligand and the sulphur atom from residue CYS277, while the red dotted line represents the hydrogen bond with the residue TRP241. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

modelling study, since this derivative incorporates in its structure more simplified analogues either in the core (cpd **6b**) or in the imidazolium region (cpds **1a** and **1b**). Among the different clusters generated by docking from different binding patterns, two were retained as they could explain the activity trends of the other imidazolium-based compounds. Interestingly, the representative poses for each cluster differed only in the orientation of the imidazolium warheads, the rest of the peptidic backbone adopting a similar conformation. Subsequently, both complexes were relaxed for 4 ns by molecular dynamics simulation in explicit water at 300 K [21,22]. The same distance between electrophilic carbon (inhibitor) and the nucleophilic sulphur atom (CYS277) was monitored (see Supporting Information). In one case, the distance remained bellow an average of 3.5 Å (Fig. 3 – Complex 1), while in the other case the ligand seems to move away from the CYS277 region (Fig. 3 – Complex 2). The first complex represents thus a plausible binding pattern for this type of ligands (Fig. 2A). The ligand adopts a bent conformation in order to present its electrophilic site closer to the catalytic CYS residue. This pose can also explain why substituents on imidazolium nitrogens would interfere with the binding, while the substituents in C4 and C5 positions would not have any strong impact as they point in the outer space of the enzyme.

When analyzing the molecular dynamics trajectory, we observed that the imidazolium warhead has privileged interactions with the TRP241 and TRP323 residues. It is important to emphasize that these TRP residues are also conserved in the case of FXIIIa, a fact that could explain the potency of this inhibitor also for this transglutaminase.

The same modelling protocol was used in the case of the dimethylsulfonium warheads (**7a**,**b**). Despite the fact that these

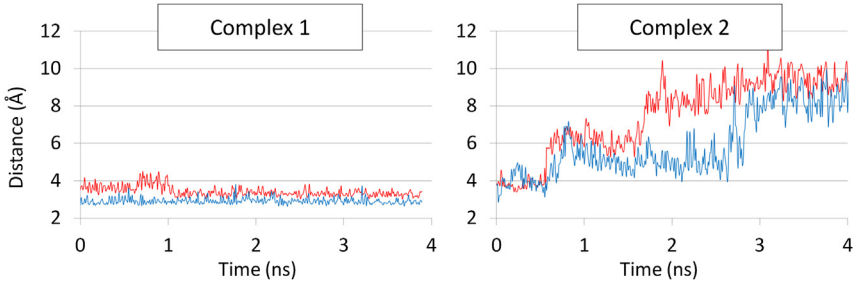


Fig. 3. Distances recorded during the molecular dynamics trajectory in case of **6a**, for selected complexes resulting from docking. In red/upper: distance between the electrophilic carbon (ligand) and the sulphur atom (CYS277); in blue/bottom: H-bond distance with TRP241. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inhibitors also adopt a bent conformation in the warhead region (Fig. 2B), the orientation is different and seems to favour a cation–pi interaction with the PHE325 residue. While the corresponding residue in FXIII seems similar (TYR372 in the case of FXIIIa vs PHE325 in the case of TG2), with the assumption that the region around the catalytic CYS residue is also conformationally highly preserved (as in TG2 case), the crystal structure of FXIII in its closed form (PDB code: 1EVU) shows that the position of this key TYR372 residue is different than that of the PHE325 in case of TG2, thus rendering difficult a similar pose of the inhibitor in the catalytic site. This could explain the specificity of inhibitors containing the dimethylsulfonium moieties for TG2.

3. Conclusion

New peptidic inhibitors incorporating a new polar warhead like imidazolium are reported. Inhibition activity of both TG2 and FXIIIa proved extremely sensitive to modification of the substituents on the imidazolium nitrogens. For example, passing from methyl to an ethyl substituent induced a complete loss of activity. When correlated with the crystal structure of TG2, these results suggested a bent conformation for the inhibitors in the warhead region. A similar bent conformation was predicted by molecular modelling in the case of another polar warhead: dialkylsulfonium. The synthesized inhibitors were tested for two members of the transglutaminase family, TG2 and FXIIIa, the dimethylsulfonium warhead rendering the inhibitors specific for TG2. Several other inhibitors from this water-soluble peptidic series are currently synthesized in our laboratory. A more complete set of ligands would help finding a general binding pattern, with the final goal of designing more potent selective TG2 inhibitors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.05.018.

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