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Design, Synthesis, and Evaluation of Gluten Peptide Analogs as Selective Inhibitors of Human Tissue Transglutaminase

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Summary

Recent studies have implicated a crucial role for tissue transglutaminase (TG2) in the pathogenesis of Celiac Sprue, a disorder of the small intestine triggered in genetically susceptible individuals by dietary exposure to gluten. Proteolytically stable peptide inhibitors of human TG2 were designed containing acivicin or alternatively 6-diazo-5-oxo-norleucine (DON) as warheads. In biochemical and cell-based assays, the best of these inhibitors, Ac-PQP-(DON)-LPF-NH_2, was considerably more potent and selective than other TG2 inhibitors reported to date. Selective pharmacological inhibition of extracellular TG2 should be useful in exploring the mechanistic implications of TG2-catalyzed modification of dietary gluten, a phenomenon of considerable relevance in Celiac Sprue.

Introduction

Celiac Sprue is a disorder of the small intestine induced by dietary exposure to gluten proteins from common food grains such as wheat, rye, and barley. Ingestion of such proteins produces flattening of the normally luxurious, rug-like, epithelial lining of the small intestine and in turn leads to clinical symptoms including fatigue, chronic diarrhea, malabsorption of nutrients, weight loss, abdominal distension, anemia, as well as a substantially enhanced risk for the development of osteoporosis and intestinal malignancies (lymphoma and carcinoma). In spite of the wide prevalence of the disease (~1:200 in most of the world's population groups), no therapeutic agents have been developed for this disease, and the only known treatment is a strict, lifelong gluten-free diet [1–3].

Recent reports have identified several short proline- and glutamine-rich sequences from wheat gluten that activate gluten-responsive T cells from Celiac patients but not from control individuals in an HLA-DQ2 (or DQ8)-dependent manner [4–7]. Importantly, most of these peptides are also substrates of the enzyme tissue transglutaminase (transglutaminase 2; tTGase; TG2, EC 2.3.2.13), which is also known to be the major focus of the autoantibody response in Celiac Sprue [8]. Moreover, TG2-mediated deamidation of these gluten peptides has been shown to specifically increase their affinity to the disease-associated HLA-DQ2 molecules, thereby enhancing their T cell stimulatory potential [4, 5, 7]. It is therefore widely believed that disease-specific T cells are rapidly activated in an HLA-DQ2-dependent fashion by TG2-mediated deamidation of gluten peptides [2]. Although the precise molecular details of this interaction in vivo remain to be eludicated, selective inhibition of TG2 in the small intestine might represent a therapeutically useful strategy for countering the immunotoxic response to dietary gluten in Celiac Sprue patients [9, 10]. The attractiveness of this proposal is supported by recent reports that transgenic mice in which the TG2 gene is inactivated are viable, phenotypically normal, and born with the expected Mendelian frequency [11, 12]. Of particular interest is the proteolytically resistant consensus sequence PQPQLPY found in many gluten proteins that is both an exceptionally good substrate of human TG2 [13, 14] and also part of the major immunodominant epitopes in gluten [4, 5, 7].

TG2 is a member of the transglutaminase family of enzymes that play important roles in diverse biological functions by selectively crosslinking proteins via γ-glutaminyl isopeptide bonds [15]. Aberrant TG2 activity is believed to play a role in neurological disorders such as Alzheimer's, Parkinson's, and Huntington's disease [16]. A variety of compounds have been used to suppress TG2-catalyzed protein crosslinking [17, 18]. However, since several of these compounds contain primary amines in addition to potential inhibitory motifs, it remains unclear whether the observed effects are due to an excess of competing amine donor or by blocking TG2 turnover. More recently, several mechanism-based active site inhibitors of guinea pig TG2 have been described by Keillor and coworkers [19–21]. These inhibitors were analogs of the widely used TG2 substrate, benzyloxycarbonyl-glutamyl-glycine (Cbz-Gln-Gly). The contribution of the peptide scaffold to inhibitor potency and selectivity remains to be explored. Moreover, the effects of these inhibitors on human TG2 or other TG2-like mammalian enzymes have not been evaluated in vitro or in cell based assays.

We observed that the specificity of human TG2 for immunogenic gluten peptides correlates well with the ground state affinity (K_M) between the enzyme and substrates [13]. Moreover, these high-specificity gluten peptides share a type II polyproline helical conformation [22]. Finally, we also observed that these peptides are remarkably resistant toward proteolysis by gastric, pancreatic, and intestinal brush border proteases [23]. Together, these observations have led us to design and evaluate analogs of these peptides as highly selective mechanism-based inhibitors of human TG2. Such inhibitors could have similar pharmacokinetic and tissue distribution properties as pathogenic gluten peptides, and

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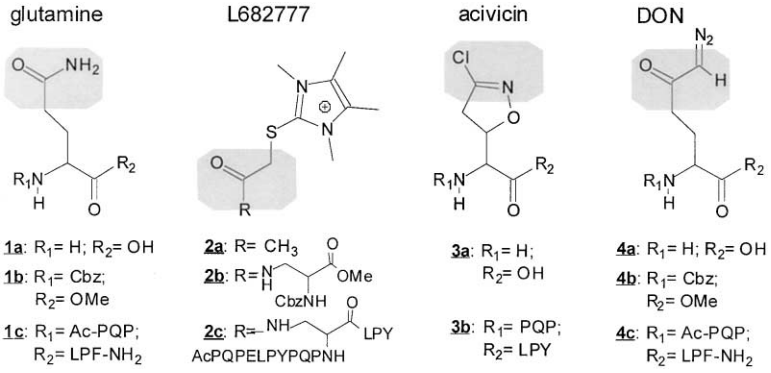


Figure 1. Reactive Glutamine Isosteres and Their Gluten Peptide Analogs

Three different reactive isoteres of glutamine were evaluated as potential inhibitors of human tissue transglutaminase. The reactive moieties are shaded.

might therefore be valuable probes of Celiac Sprue pathogenesis.

Results

Design, Synthesis, and In Vitro Evaluation of Selective Inhibitors of Human TG2

We sought to replace the reactive glutamine (underlined) of the high-affinity TG2 substrate, PQPQLPY [13], with reactive glutamine isosteres shown in Figure 1. We first evaluated analogs containing a tetramethyl thioimidazolyl moiety. A prototype compound of this class, L682777 (**2a**, Figure 1), is one of the most potent inhibitors of the transglutaminase family of enzymes [24], including human TG2, as demonstrated in this study ($k_{inh}/K_I = 1.4\ min^{-1}\ mM^{-1}$). We therefore introduced this compound in place of the γ-carbonyl group of glutamine embedded in a low-affinity Cbz-X-OMe and a high-affinity AcPQPELPYPQP-X-LPY scaffold. The specificity of the resulting tetramethyl thioimidazolium inhibitors **2b** and **2c** was reduced compared to L682777 (data not shown), in part because of weaker binding to the TG2 active site. This decrease in specificity suggests that the carbonyl group of L682777 might be positioned quite differently in the active site of human TG2 than the γ-carbonylamide group of a reactive glutamine in TG2 substrates.

We therefore considered the naturally occurring glutamine analogs acivicin (Aci) **3a** and 6-diazo-5-oxo-norleucine (DON) **4a**, both originally isolated as antibiotics from *Streptomyces* strains. Both amino acids are bona fide glutamine isosteres, as judged by their potent inhibition of γ-glutamyl transpeptidase (γ-gTP, EC 2.3.2.2) [25]. γ-gTP is structurally and mechanistically related to TG2, and also catalyzes a similar transamidation reaction. In addition the diazoketone moiety present in DON is a well-established inhibitory motif of cysteine proteases [26]. At high concentrations (≈ 100 mM), both acivicin and DON inactivated human TG2, suggesting their potential use as "warheads" in an appropriate high-affinity peptide context (Table 1).

To test this hypothesis, commercially available acivicin was protected with the fluorenyl-methoxycarbonyl (FMOC) group and incorporated into the PQP-Aci-LPY **3b** sequence by standard automated solid phase peptide synthesis (Figure 2A). The HPLC-purified product was incubated with recombinant human TG2 [13] together with varying amounts of the substrate Cbz-Gln-Gly ($K_M = 5.9$ mM). The inhibition parameters were obtained by progress curve analysis [27] using a continuous glutamate dehydrogenase coupled assay [28]. As summarized in Table 1, derivatization of acivicin by the flanking PQP and LPY tripeptides increased its specificity (k_{inh}/K_I) 100-fold compared to unmodified acivicin. This result demonstrated that high-affinity peptide sequences could be exploited to enhance the specificity of small molecule inhibitors of human TG2. However, comparison with the kinetic parameters of unmodified glutamine as a TG2 substrate versus PQPQLPY revealed that the large difference in substrate specificity $[(k_{cat}/K_M)^{Gln} < 10^{-4} \times (k_{cat}/K_M)^{PQPQLPY}]$ was only partially reflected in the observed difference in inhibitor specificity $[(k_{inh}/K_I)^{Aci} \approx 0.01 \times (k_{inh}/K_I)^{PQPAciLPY}]$.

A possible reason for the suboptimal cooperativity between acivicin and the flanking peptides could be that the constrained dihydro isoxazole ring assumes an unfavorable conformation upon binding to the active site that cannot be alleviated as efficiently in the context of the peptide as compared to free acivicin. Indeed, earlier studies on the carbonyl amide donor requirements of guinea pig TG2 indicated a preference for an unsubstituted extended -CH_2-CH_2- chain adjacent to the carbonyl amide [17]. To address this possibility, the unbranched 6-diazo-5-oxo-norleucine was incorporated into a low-affinity scaffold, yielding Cbz-DON-OMe **4b**, and a high-affinity scaffold, yielding Ac-PQP-DON-LPF-NH_2 **4c**. (Relative to PQPQLPY, N-terminal acetylation, C-terminal amidation or a Y→F substitution do not have a significant impact on k_{cat} or K_M [Table 1]). To avoid cyclization of the γ-glutamyl moiety, the N-Cbz-5-oxazolidinone-protected glutamate was activated by thionyl chloride and reacted with diazomethane [29] yielding **4b** by subsequent methanolysis (Figure 2B). Activation of the central glutamate in Ac-PQPELPF-NH_2 as a mixed anhydride followed by treatment with diazomethane provided **4c** after HPLC purification (Figure 2C).

Notably, the second-order inactivation constants (k_{inh}/K_I) were 4500-fold and 1.5×10^7-fold higher for **4b** and **4c**, respectively, compared to unmodified DON (Table 1). As shown in Figure 3A, **4c** rapidly inactivates TG2 at a concentration of 1 μM even in the presence of a high substrate concentration ($[S] \geq 10 \times K_M$). The inactivated enzyme could not be reactivated even after overnight incubation in the absence of free inhibitor or in the presence of an alternative nucleophile amine donor such as

Table 1. Kinetic Parameters of Catalysis and Inhibition of Tissue Transglutaminase by Reactive Glutamine Peptide Analogs

Reactive Motif	Gln			Aci			DON		
Scaffold	k_{cat} [min^{-1}]	K_M [M]	k_{cat}/K_M [$min^{-1}M^{-1}$]	k_{inh} [min^{-1}]	K_I [M]	k_{inh}/K_I [$min^{-1}M^{-1}$]	k_{inh} [min^{-1}]	K_I [M]	k_{inh}/K_I [$min^{-1}M^{-1}$]
H-**X**-OH	–	>0.2	≤ 2	0.015	0.087	0.17	0.025	0.13	0.2
Cbz-**X**-OMe	–	>0.03	90	–	–	–	0.12	1.35×10^{-4}	890
PQP-**X**-LPY	28[a]	3×10^{-4}[a]	9.3×10^{4}[a]	0.014	7.8×10^{-4}	18	–	–	–
Ac-PQP-**X**-LPF-NH_2	40	4×10^{-4}	9.7×10^{4}	–	–	–	0.2	7×10^{-8}	2.9×10^{6}

The reactive glutamine (-**X**-) in the peptide substrate was substituted by the inhibitory residue acivicin (Aci) or 6-diazo-5-oxo-norleucine (DON). Catalytic parameters for deamidation of glutamine-containing analogs were obtained via a coupled NADH/glutamate dehydrogenase assay. Inhibitory constants for acivicin and 6-diazo-5-oxo-norleucine were obtained by progress curve analysis using varying amounts of the reference substrate Cbz-Gln-Gly.
[a]Adopted from reference [13].

Ac-Lys-OMe (data not shown), suggesting this inhibition to be either irreversible or slow and tight binding. The relative specificity (k_{inh}/K_I) of inhibitors **4a**, **4b**, and **4c** correlated well with the relative specificity (k_{cat}/K_M) of TG2 for the corresponding substrates **1a**, **1b**, and **1c** (Table 1). As predicted, the enhanced specificity of the DON-containing peptides was primarily due to lower K_I values; the first-order inactivation constant (k_{inh}) remained relatively unchanged for **4a**, **4b**, and **4c**. Finally, in agreement with the design of isosteric analogs, TG2 inhibition by Cbz-DON-OMe **4b** was competitive with respect to the reference Cbz-Gln-Gly substrate (Figure 3B). Together, these observations demonstrated that the diazoketone moiety of DON is an excellent isostere of glutamine in the active site of human TG2.

Selectivity of DON-Containing Peptide Inhibitors

It was also anticipated that conversion of DON into peptidic derivatives would alter the specificity of this warhead for other competing enzymes that are likely to be present in the celiac gut under physiological conditions. For example, whereas the related enzyme γ-glutamyl transpeptidase (γ-gTP) was efficiently inactivated by free DON **4a** with a second-order rate constant ($k_{inh}/K_I)^{DON} = 120\ min^{-1}M^{-1}$, its inactivation by Cbz-DON-OMe **4b** reduced to $(k_{inh}/K_I)^{Cbz\text{-}DON\text{-}OMe} = 0.03\ min^{-1}mM^{-1}$. Thus, the Cbz- and methoxy-protection apparently reversed the preference of the DON warhead from γ-gTP to TG2, resulting in a relative selectivity of $(k_{inh}/K_I)^{TG2}/(k_{inh}/K_I)^{\gamma\text{-}gTP} \approx 3 \times 10^4$ for Cbz-DON-OMe **4b**. A similar reversal in selectivity was found for Ac-PQP-DON-LPF-NH_2 **4c**. These results are consistent with the known specificity of γ-gTP [30, 31], and suggest that DON-containing peptide inhibitors may have the necessary specificity to be effective in cell-based and in vivo assays.

Evaluation of TG2 Inhibitors in a Model for Enterocyte Differentiation

As a final test of the potential utility of these inhibitors as probes of the role of TG2 in intestinal biology, an assay for differentiation of human T84 epithelial cells in three-dimensional collagen gel cultures containing IMR-90 fibroblasts was used [32, 33]. In this assay, T84 cells are cultured within type I collagen gel together with fibroblasts without cell-to-cell contact. In such a coculture model for enterocyte differentiation, a majority of T84 colonies organize into luminal formations with well-deposited basement membranes, as well as morphologically and enzymatically mature brush border membranes. Dose-dependent inhibition of differentiation was observed by both L682777 and Ac-PQP-(DON)-LPF-NH_2 over a range of 1–100 μM and 0.1–10 μM inhibitor con-

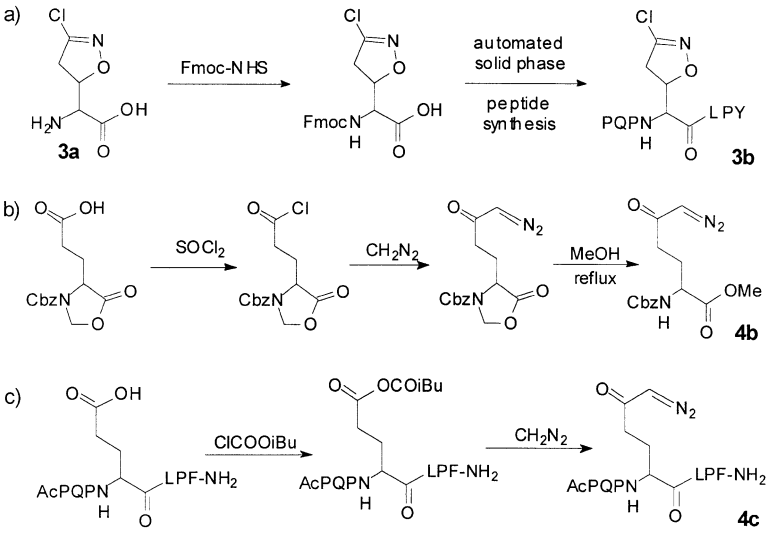


Figure 2. Synthesis of Protected or Peptide-Bound Reactive Glutamine Isosteres

For details, see Experimental Procedures.

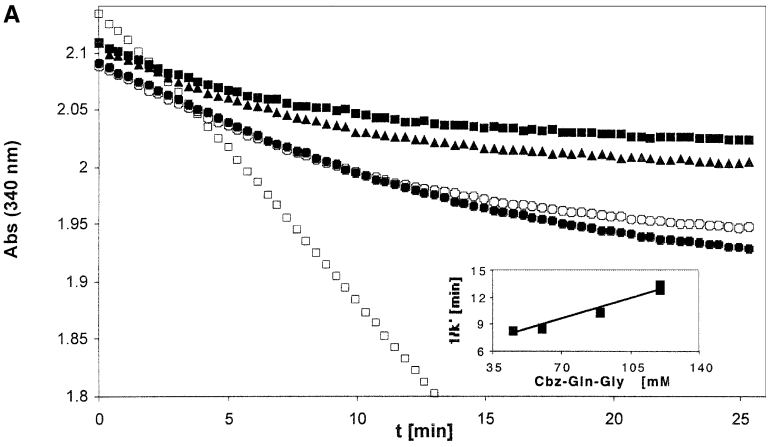
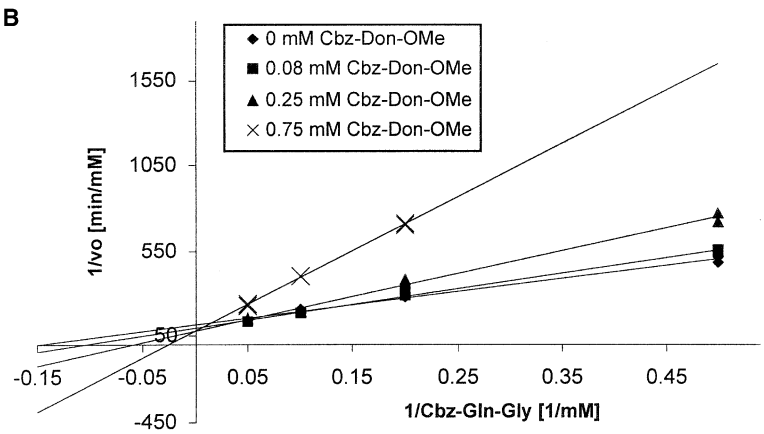


Figure 3. Irreversible Inhibition of Tissue Transglutaminase by 6-Diazo-5-Oxo-Norleucine-Containing Compounds

(A) Progress curve analysis of tissue transglutaminase incubated with 1 μM Ac-PQP-DON-LPF-NH_2 **4c** and 45 mM (closed squares), 60 mM (closed triangles), 90 mM (open circles), or 120 mM (closed circles) of the substrate Cbz-Gln-Gly. Incubation with 45 mM Cbz-Gln-Gly alone without inhibitor (open squares). Inset: progress curves were nonlinear least-squares fitted, as described in the Experimental Procedures section, to calculate the inhibition constants.

(B) Double-reciprocal plot of competitive inhibition of tissue transglutaminase by Cbz-DON-OMe **4b**. Initial velocities (v_0) of Cbz-Gln-Gly deamidation in the presence of the indicated amounts of this inhibitor were obtained by nonlinear least-squares fitting of the corresponding progress curves.



centrations, respectively (Figure 4). In the same concentration range for both inhibitors tested, significant cytotoxicity was neither observed toward T84 cells nor fibroblasts. These findings are in contrast with other nonspecific small molecule TG2 inhibitors tested (T.H., unpublished data), such as cystamine dihydrochloride [34] or factor XIIIa fragment 72–97 [35]. Our results are also reminiscent of the effect of anti-TG2 autoantibodies from Celiac Sprue patients on T84 differentiation [33] and suggest that the primary target of the peptide inhibitor is extracellular TG2. It should be noted that although enterocyte differentiation is inhibited in this assay, presumably this arises due to inactivation of TG2 on both the mucosal and serosal sides of enterocytes. In con-

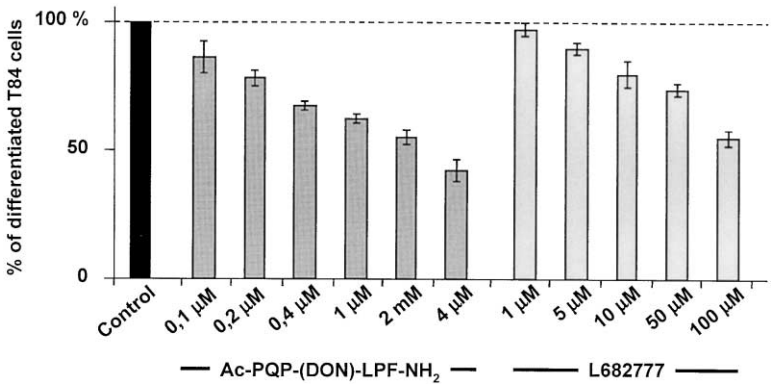


Figure 4. Inhibition of T84 Human Intestinal Epithelial Cell Differentiation

Inhibition of T84 differentiation was performed on cells from 11 days cultures and is expressed as percent differentiated T84 cell colonies relative to an equivalent DMSO control. The columns represent the average of three independent sets of experiments with two replicates, and the bars represent the standard deviations.

trast, serosal side TG2 in the intact intestine is inaccessible to both peptide substrates and inhibitors, and should be able to facilitate normal enterocyte differentiation. Further animal studies with these inhibitors could help address the role of TG2 localization in differentiation of the small intestinal epithelium.

Significance

In this study, acivicin and especially 6-diazo-5-oxo-norleucine (DON) were identified as potent inhibitory motifs of human TG2. The excellent molecular mimicry of glutamine by DON in the active site of TG2 facilitated the conversion of proteolytically stable high-affinity TG2 substrates into selective inhibitors in a predictable manner. The best inhibitor in this series, Ac-PQP-DON-LPF-NH_2, is capable of inactivating 90% of TG2 activity within 20 min at submicromolar concentrations. (At higher concentrations complete inhibition can be achieved in shorter times.) It has substantially greater selectivity toward TG2 as compared to similar enzymes such as γ-glutamyl transpeptidase. Additionally, the absorption and tissue-level distribution properties of such gluten-like inhibitors are likely to resemble those of the inflammatory gluten peptides themselves. As such, it may be possible to "follow the gluten trail" in the preferential targeting of oral gluten analogs to intestinal TG2. We note that, although an enteric coating would be required to insulate DON-containing inhibitors from the acidic environment of the stomach, the inhibitors should be stable in the neutral pH environment of the small intestine. And finally, at micromolar concentrations, they can effectively inhibit TG2-mediated differentiation of T84 cells, an established model of intestinal enterocyte maturation.

TG2 inhibitors that mimic proteolytically stable gluten peptides may not only be useful chemical probes of the the role of extracellular TG2 in small intestinal biology but also in establishing the precise role of TG2 in Celiac Sprue. Finally, since aberrant TG2 activity has also been implicated in certain dermatological and neurodegenerative disorders [15, 16], the inhibitors reported here might also serve as useful probes of the role of TG2 in these diseases.

Experimental Procedures

General

All reagents for chemical synthesis were obtained from Aldrich (MO); all reagents and enzymes for biochemical assays were from Sigma (MO). Peptides were synthesized by the Stanford Protein and Nucleic Acid facility on a Perkin Elmer/ABI 433A using standard HBTU-coupling/TFA deprotection and purified by preparative reversed phase HPLC on a Beckman Ultrasphere C18 column (15 × 2.54 cm). NMR spectra were acquired on a Varian Gemini-200 or Varian Inova-500 with tetramethylsilane as a standard.

Synthesis of 1,3,4,5-Tetramethyl-2-[(2-Oxopropyl)thio]imidazolium Chloride (L682777) 2a

0.5 g (3.2 mmol) of 1-thio-2,3,4,5-tetramethyl-imidazole [36] in 3 ml chloroform was reacted with 300 μl (3.75 mmol) chloroacetone overnight at room temperature. After rotary evaporation, recrystallization from 10 ml isopropanol:hexane (1:1) yielded 0.65 g (2.6 mmol, 70%) of dry, white needles. mp = 177°C.

^{1}H ($CDCl_3$, 200 MHz) = 4.06 $SCH_2C(O)$ (2H, s); 3.53 CH_3N (6H, s); 1.86 CH_3C (6H, s); 1.83 $C(O)CH_3$ (3H, s). ^{13}C ($CDCl_3$, 200 MHz) = 200.3, 135.3, 128.5, 45.1, 33.3, 28.2, 8.6.

m/z (ESI) = 213.1 (100%)$[M\text{-}Cl]^+$; 460.7 (10%)$[2M\text{-}Cl]^+$.

Synthesis of Compound 2b

3.7 g (15.5 mmol) of 2-N-benzyloxycarbonyl-diamino propionic acid (Cbz-Dap-OH, Bachem) in 35 ml methanol was carboxymethylated by treatment with 10 ml trimethylsilyl chloride overnight at room temperature. Solvent and excess reactant were removed by rotary evaporation, and the product was recrystallized from methanol to yield 3.5 g (80%) of pure, dry Cbz-Dap-OMe (m/z = 253.1). To 0.98 g (3.9 mmol) of the latter intermediate resuspended in 15 ml dry acetonitrile and 0.95 ml triethylamine was added 0.95 g (3.4 mmol) bromoacetic acid anhydride and 10 mg 4-dimethylaminopyridine. After stirring for 2 hr, the solvent was removed by rotary evaporation. The resulting residue was dissolved in ethyl acetate and extracted with 2 vol each of aqueous saturated solutions of $NaHCO_3$, $NaHSO_4$, and NaCl. The organic phase was concentrated and recrystallization from isopropanol:hexane yielded 0.43 g (35%) of the desired 3-N-bromoacetyl-2-N-Cbz-propionic acid methyl ester [Cbz-Dap(AcBr)-OMe]. 285 mg (0.75 mmol) of this compound and 120 mg (0.77 mmol) of 1-thio-2,3,4,5-tetramethyl-imidazole [36] in 1 ml chloroform was stirred overnight at room temperature. The raw product was concentrated purified by silica gel flash chromatography with $CHCl_3$:MeOH:TFA (9:1:0.1) as eluent yielding 490 mg of **2b** as a red glass-like substance after drying in vacuo.

R_f ($CHCl_3$:MeOH:TFA = 9:1:0.1) = 0.09

^{1}H (d^6-DMSO, 500 MHz) = 8.52 NH (1H, t, J = 5.5 Hz); 7.72 NH (1H, d, J = 8.5 Hz); 7.35 ArH (5H, m); 5.05 $ArCH_2$ (2H, s); 4.15 α-CH (1H, m); 3.75 NCH_3 (6H, s); 3.63 SCH_2 and OCH_3 (5H, s); 3.49–3.5 β-CH (1H, m); 3.25–3.27 β-CH′ (1H, m); 2.29 NCH_3 (6H, s).

^{13}C (d^6-DMSO, 500 MHz) = 171.5, 168.1, 156.7, 137.4, 136.8, 129.9, 129.1, 128.5, 74.4, 66.4, 54.1, 52,8, 37.6, 34.1, 9.4.

m/z (ESI) = 449.3 (100%) $[M]^+$.

Synthesis of Compound 2c

Ac-PQPELPYPQP-Dap-LPY was synthesized by solid phase peptide synthesis using Fmoc-Dap(Boc)-OH (Bachem) to introduce the diaminopropionic acid residue. 110 mg (66 μmol) of the deprotected HPLC-purified peptide were dissolved in 660 μl of a 1M triethylamine bicarbonate buffer (Fluka) and reacted with 400 μl of a 1 M solution of bromoacetic acid N-hydroxysuccinimide ester in DMF at 4°C. After 2 hr, the reaction was quenched by addition of 80 μl of TFA, and the bromoacetylated intermediate was purified by reverse phase HPLC using a water: acetonitrile: 0.1% TFA gradient. Lyophilization of the major product eluting at 31% acetonitrile yielded 33.5 mg bromoacetylated peptide with a the expected λ_{max} = 275 nm, mass isotope distribution and fragmentation pattern (m/z $[M+H]^+$ = 1786.3 [60%], 1787.3 [65%], 1788.5 [100%], 1749 [60%], 1790.3 [40%]).

28.3 mg of Ac-PQPELPYPQP-Dap(AcBr)-LPY was reacted overnight with 26.2 mg (170 μmol) 1-thio-2,3,4,5-tetramethyl-imidazole [36] in 400 μl $CHCl_3$:DMF (1:1) at room temperature. The solvent was concentrated by rotary evaporation, the peptide precipitated by addition of ice-cold ether, and the resulting residue was purified by reverse phase HPLC using a water: acetonitrile: 0.1% TFA gradient. The product **2c** eluting at 32.5% acetonitrile was lyophilized yielding 16.2 mg (8.7 μmol) with an ϵ_{269}^{max} = 9,600 M^{-1} cm^{-1}.

LC-MS: m/z $[M]^+$ = 1862.9 (70%), 1863.8 (100%), 1864.8 (50%), 1865.9 (15%).

Synthesis of Fmoc-Acivicin

3.1 ml of a 0.75 M solution of Fmoc-N-hydroxysuccinimide in acetone were added to 0.4 g acivicin (L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid, 2.25 mmol; Biomol, PA) dissolved in 3.1 ml of a 10% Na_2CO_3 aqueous solution. The slurry was stirred for 1 hr at room temperature, and the pH was maintained at 9.0 by addition of Na_2CO_3. The solvent was removed by rotary evaporation, the residual solid was dissolved in 0.6 M HCl, extracted with ethyl acetate, and the combined organic phases were concentrated to a yellow oil. Recrystallization from ethyl acetate: hexane yielded 0.62 g (1.55 mmol, 70%) of the desired product as white crystals.

R_f (CH_2Cl_2: iPrOH: AcOH = 100: 3: 1) = 0.3

^1H (d^6-acetone, 200MHz) = 7.87 ArH (2H, d, J = 7.4 Hz); 7.73 ArH (2H, d, J = 7 Hz); 7.28–7.48 ArH (4H, m); 7.17 NH (1H, d, J = 8 Hz); 5.22 β-CH (1H, m); 4.66 α-CH (1H, dd, J_1 = 8.7 Hz, J_2 = 4.5 Hz); 4.2–4.4 (3H); 3.6–3.4 (2H).

^{13}C (d^6-acetone, 200 MHz) = 213.4, 207.7, 145.1, 128.6, 128.0, 126.2, 120.9, 82.9, 67.6, 56.9, 47.9, 41.0.

m/z (ESI) = 401.3 (40%) $[M+H]^+$, 423.4 (100%) $[M+Na]^+$, 424.2 (90%) $[M+Na]^+$, 425.3 (35%) $[M+Na]^+$.

Synthesis of Pro-Gln-Pro-Aci-Leu-Pro-Tyr 3b

PQPAciLPY was assembled by standard Fmoc solid phase chemistry using Fmoc-acivicin and commercially available building blocks in a 25 μmol scale on a Symphony/Multiplex automated peptide synthesizer. After cleavage/deprotection with TFA, preparative reverse phase HPLC on a Beckman Ultrasphere C18 column (15 × 2.54 cm) with a water: acetonitrile: 0.1% TFA gradient yielded 4 OD_{275} (3.4 μmol, 14% yield based on one tyrosine with ϵ_{272} = 1200 $cm^{-1}M^{-1}$) of the desired peptide. The product eluted at 24.5% acetonitrile.

LC-MS: $[M+H]^+$ = 874.6 g/mol.

Synthesis of Cbz-DON-OMe 4b

6 g (20 mmol) S-(+)-3-benzyloxzcarbonyl-5-oxo-4-oxazolidine propionic acid in 37 ml ethanol-free chloroform was reacted with 1.8 ml thionyl chloride for 3 hr at room temperature [29]. After rotary evaporation, the residual oil was diluted with additional chloroform, which was removed under reduced pressure. To the resulting oil, dissolved at 0°C in 15 ml THF, was added drop-wise a saturated, ethanol-free solution of diazomethane in ice-cold ether until gas formation ceased and an intense yellow color persisted. The latter solution was prepared fresh from diazald/KOH, as described by the manufacturer (Aldrich). The solvent was removed by rotary evaporation, and the residue was redissolved in methanol and refluxed for 10 min after addition of sodium bicarbonate. The solvent was removed by rotary evaporation, and the resulting oil was extracted with ethyl acetate and a saturated aqueous solution of sodium bicarbonate. The pooled organic phases were dried over sodium sulfate, concentrated, and purified by silica gel flash chromatography with hexane:ethyl acetate:triethylamine (1:1:0.01) as eluent. Additional reverse phase HPLC purification on a Beckman Ultrasphere C18 column (15 × 2.54 cm) with a water: acetonitrile: 0.1% ammonium hydrogen carbonate gradient provided 85 mg (0.26 mmol) of pure product, eluting at 38.5% acetonitrile. The product was dried in vacuo.

R_f (hexane: ethyl acetate: triethylamine = 1: 1: 0.01) = 0.2.

^1H (d^6-DMSO, 500MHz) = 7.77 NH (1H, d, J = 9.6 Hz); ArH (2H, d, J = 7.4 Hz); 7.34-7.30 ArH (5H, m); 6.07 CHN_2 (1H, s); 5.04 $PhCH_2O$ (3H, s); 4.08-4.01 α-CH (1H, m); 3.63 OCH_3 (3H, s); 2.40 γ-CH_2 (2H, m); 1.97 β-CH (1H, m), 1.78 β-CH′ (1H, m).

m/z (ESI) = 341.9 (100%)$[M+Na]^+$; 314.1 (22%)$[M+Na\text{-}N_2]^+$.

Synthesis of Ac-Pro-Gln-Pro-DON-Leu-Pro-Phe-NH_2 4c

72 mg (8.3 μmol) of HPLC-purified, lyophilized Ac-PQPELPF-NH_2 in 1 ml THF and 15 μl (135 μmol) N-methyl morpholine were mixed with 13 μl (100 μmol) isobutyl chloroformate at 0°C, immediately followed by addition of up to 0.5 mol of a saturated diazomethane solution in dry ether generated from Diazald as described by the supplier. After 1 hr, the solvents were evaporated, the residual solid was extracted with ethyl acetate and a 5% aqueous solution of NH_4HCO_3, and the combined aqueous phases were concentrated by rotary evaporation. The crude product was purified by semipreparative reversed phase HPLC on a Vydac 259VHP810 RP polymer column (d = 1 cm) using a 0.1 M triethylamine hydrogen carbonate buffer (Fluka) with increasing percentage of acetonitrile as mobile phase. The UV_{280}-active fractions eluting at 18.9% acetonitrile were pooled and lyophilized yielding 19.4 OD_{275} of HPLC-pure **4c** (1.7 μmol, based on an $\epsilon^{DON}{}_{275}$ = 11,700 $cm^{-1}M^{-1}$ as determined for DON (Bachem) in 10 mM Tris-HCl pH = 8.5).

m/z (ESI) = 914.2 (100%) $[M+Na]^+$; 886.5 (75%) $[M+Na\text{-}N_2]^+$.

Determination of TG2 Activity

TG2 was assayed routinely as described [13, 28], in a 1 ml or 150 μl reaction mixture containing 200 mM MOPS pH = 7.1, 5 mM $CaCl_2$, 1 mM EDTA, 10 mM α-ketoglutarate, 18 U/ml glutamate dehydrogenase (Biozyme, CA), 0.4 mM NADH, 3.3% DMSO, 0.5 μM TG2, and the indicated amount of substrate. The reaction of the prewarmed and equilibrated components was started by addition of TG2 and was monitored spectrophotometrically by following the NADH consumption at 340 nm (ϵ_{340} = 6220 $cm^{-1}M^{-1}$) and 30°C from 2.5 to 25 min. Measurements were performed in duplicate using 2, 5, 10, and 20 mM Cbz-Gln-Gly as a sodium salt, 19–150 mM glutamine, 1–10 mM Cbz-Gln-OMe (Bachem), or 0–1.2 mM Ac-PQPQLPF-NH_2.

Inhibition of TG2

In order to quantify inhibition of human TG2 by acivicin **3a** (50 mM), PQP**Aci**LPY **3b** (0.45 mM), or Cbz-DON-OMe **4b** (0, 0.08, 0.25, or 0.75 mM), the enzyme was incubated with appropriate concentrations of the inhibitors in the TG2 reaction buffer (above). After 2.5 min NADH consumption was monitored in the presence of 2, 5, 10, or 20 mM Cbz-Gln-Gly·Na. The progress curves were fitted to the following equation which describes product formation as decrease of absorption at λ = 340 nm with simultaneous exponential decay of enzyme as modified from [27]: $Abs = Abs_0 - v_0 \cdot 6.22\ mM^{-1} \cdot (1\text{-}e^{-k't})/k'$. Substrate dependence of v_0 and k′ yielded the kinetic parameters k_{inh} and K_I. Inactivation by Ac-PQP-DON-LPF-NH_2 **4c** (1 μM) was measured at 0.3 μM TG2 in the presence of 45, 60, 90, and 120 mM Cbz-Gln-Gly·Na to compete efficiently with the inhibitor. Due to the high absorption of DON at λ = 340 nm, inhibition of TG2 (25 μM) by free DON **4a** (0, 30, 100, and 300 mM) was measured discontinuously in 200 mM MOPS, pH = 7.1, 5 mM $CaCl_2$, 1 mM ETDA at 30°C. Residual activity of 20 μl aliquots withdrawn at t = 3–112 min were determined by dilution into 1 ml of assay buffer using 10 mM Cbz-Gln-Gly·Na. Inhibition by 0–8 μM L682777 **2a** was determined with 7.8 mM Cbz-Gln-Gly·Na.

Confirmation of Irreversible Inactivation

10 μM TG2 in 200 mM MOPS, pH = 7.1, 5 mM $CaCl_2$, 1 mM EDTA, 3.3% DMSO was treated with or without 25 μM Ac-PQP-DON-LPF-NH_2 **4c** for 90 min at 30°C. Successive gel filtration over a Pharmacia NAP-5 and PD-10 column equilibrated in the same buffer was used to remove excess inhibitor. Residual TG2 activity in the resulting solutions with or without initial inhibitor was measured after 150, 300, and 1000 min at 30°C. Additionally, a final concentration of 0.2 mM N-acetyl-lysine-methyl ester (Ac-Lys-OMe) was added after gel filtration, and residual TG2 activity was measured after 75 and 1000 min.

Inhibition of γ-glutamyl Transpeptidase (γ-gTP)

Progressive inhibition of γ-gTP (type II from bovine kidney) by 0–25 mM DON was assayed in 200 mM MOPS, pH = 7.1, using 20 mM Gly-Gly, 1 mM L-γ-glutamyl-p-nitroanilide, for 15 min at 30°C [25]. The reaction was started by addition of γ-gTP to a final concentration of 40 μU/μl. Product formation of the liberated p-nitroanilide (ϵ_{410} = 8,800 cm^{-1} M^{-1}) was fitted to a competitive, exponential inhibition [27].

220 μU/μl γ-gTP in 200 mM MOPS, pH = 7.1, 5.5% DMSO were treated with 0–5 mM Cbz-DON-OMe **4b** for 1300 min at 30°C. Residual γ-gTP activity was determined spectroscopically at 410 nm after 1:11 dilution with 1.1 mM L-γ-glutamyl-p-nitroanilide corroborating linear concentration dependence. Similarly, treatment with 20 μM Ac-PQP-DON-LPF-NH_2 **4c** for 1200 min resulted in 15% γ-gTP activity compared to uninhibited control.

Inhibition of T84 Intestinal Cell Differentiation

The ability of Ac-PQP-(DON)-LPF-NH_2 and L682777 to inhibit intestinal T84 epithelial cell differentiation was tested using a previously described three-dimensional epithelial-mesenchymal cell coculture model [32]. This model has also been used to establish a role for extracellular TG2 in this differentiation process through the use of anti-TG2 antibodies derived from Celiac Sprue patients [33]. For three-dimensional cocultures 5 × 10^5 T84 epithelial cells were suspended in 1 ml of ice-cold type I collagen solution (8 vol) supplemented with 10× concentrated RPMI (1 vol) (GIBCO-BRL) and 7.5% $NaHCO_3$ (1 vol) and layered onto Nunclon 24-well plates (Nunc, Roskilde, Denmark). After a cell-free collagen layer fibroblast suspension (5 × 10^5 cells/ml) in 1:1 mixture of supplemented DMEM/F12 (1:1) (GIBCO-BRL) and BME (GIBCO-BRL) were added and grown as

monolayer, cultures were kept in a humidified 5% CO_2 atmosphere at 37°C for 8–11 days. The inhibitors Ac-PQP-(DON)-LPF-NH_2 and L682777 were added into the culture medium at final concentrations ranging from 0.1 to 8 μM and 1 to 100 μM, respectively. Equally diluted DMSO served as control, and the results are expressed as percentage of differentiated T84 cell colonies relative to an equivalent DMSO control. Assays were performed with two replicates in three independent sets of experiments.

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