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An Unprecedented Dual Antagonist and Agonist of Human Transglutaminase 2

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Abstract

Transglutaminase 2 (TG2) is a ubiquitously expressed, Ca²⁺-activated extracellular enzyme in mammals that is maintained in a catalytically dormant state by multiple mechanisms. Although its precise physiological role in the extracellular matrix remains unclear, aberrantly up-regulated TG2 activity is a hallmark of several maladies, including celiac disease. Previously, we reported the discovery of a class of acylideneoxindoles as potent, reversible inhibitors of human TG2. Detailed analysis of one of those inhibitors (CK-IV-55) led to an unprecedented and striking observation. Whereas this compound was a non-competitive inhibitor ($3.3 \pm 0.9 \mu\text{M}$) of human TG2 at saturating Ca²⁺ concentrations, it activated TG2 in the presence of sub-saturating but physiologically relevant Ca²⁺ concentrations (0.5–0.7 mM). This finding was validated in a cellular model of TG2 activation and inhibition. Mutant TG2 analysis suggested that CK-IV-55 and its analogs bound to a low-affinity Ca²⁺ binding site on the catalytic core of TG2. A mechanistic model for the dual agonistic/antagonistic action of CK-IV-55 on TG2 is presented, and the pathophysiological implications of basal activation of intestinal TG2 by small molecules are discussed.

Graphical Abstract

CK-IV-55 can activate/inhibit human transglutaminase 2.

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Transglutaminase 2 (TG2) is the most prevalent member of the mammalian transglutaminase family, with abundant intracellular as well as extracellular expression in most organs. It catalyzes transamidation or deamidation of Gln residues in protein and peptidic substrates, and is regulated by several post-translational mechanisms¹. In the absence of guanine nucleotides and presence of Ca^{2+} , TG2 adopts an open, catalytically active conformation^{2,3}. Reduction of an intramolecular, vicinal disulfide bond is also required for enzymatic activity^{4,5}. Whereas the precise biological role of TG2 remains unclear, the protein may play an important role in the pathogenesis of a variety of human diseases. For example, deamidation of selected Gln residues in proteolytically stable peptides derived from dietary gluten is believed to underlie celiac disease pathogenesis⁶⁻⁸. Aberrant TG2 expression and activity is also implicated in the pathogenesis of other disorders, such as cancer, Alzheimer's disease, Parkinson's disease, Huntington's disease, and cystic fibrosis⁹⁻¹³. Thus, TG2 is a target of interest for the development of inhibitors.

In 2011, we identified acylideneoxindoles as a new class of reversible inhibitors of TG2¹⁴. A subset of these molecules exhibited mixed inhibitory behavior, suggesting that this class of TG2 modulating agents bound to an allosteric site on the enzyme. Here, we demonstrate that, at low concentrations of free Ca^{2+} , some of these compounds can augment the activity of TG2. One molecule, CK-IV-55, was capable of activating TG2 in the extracellular matrix of cultured WI-38 fibroblast cells.

The allele of human TG2¹⁵ used in this study is the V224 variant. Although our previous studies utilized the G224 form of recombinant human TG2¹⁶, sequence analysis has demonstrated that Val is the most common residue at this position. Moreover, the V224 variant is reported to have higher Ca^{2+} -sensitivity and activity¹⁵.

To reassess the dependence of TG2 on its allosteric regulators, GTP and Ca^{2+} , a coupled enzymatic assay developed by Keillor and Day¹⁷ was employed. In brief, TG2 catalyzes the deamidation of the protected dipeptide substrate Cbz-Gln-Gly (ZQG), releasing ammonia, which is used in the glutamate dehydrogenase-dependent conversion of α -ketoglutarate to glutamate. The latter step utilizes the cofactor, NADH, allowing the reaction to be monitored continuously at 340 nm. Under saturating Ca^{2+} concentrations, the k_{cat} and K_{M} of the V224 variant were $16.8 \pm 0.8 \text{ min}^{-1}$ and $4.7 \pm 0.6 \text{ mM}$, respectively, verifying a higher catalytic efficiency compared to the G224 form of the enzyme (Figure S1, Table S1). Lineweaver-Burk analysis (Figure 1A) revealed that Ca^{2+} acted predominantly as a competitive activator of V224 TG2 ($K_{\text{a}} = 0.6 \pm 0.3 \text{ mM}$) in contrast to mixed activation of the G224 variant¹⁸, and that the stable GTP analog, GMP-PNP, was a competitive inhibitor ($K_{\text{i}} = 27 \pm 9 \text{ }\mu\text{M}$) of TG2 (Figure 1B).

As a first step toward broader biological use of our acylideneoxindole inhibitors, we attempted to block the activity of extracellular TG2 in tissue culture. WI-38 fibroblasts were grown to near-confluence, and exposed to either vehicle (DMSO) or selected inhibitors for 3 h. Cumulative TG2 activity during this period was measured by incorporation of 5-(biotinamido)-pentylamine (5-BP) into proteins of the extracellular matrix, as shown before¹⁹⁻²¹. Surprisingly, addition of $1 \text{ }\mu\text{M}$ CK-IV-55 (Table 1) to this assay system resulted in activation of extracellular TG2 (Figure 2). At higher concentrations of CK-IV-55 (10

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μM), TG2 activity was suppressed compared to the 1 μM case. Similar observations were made with a close structural analog, CK-IV-67 (Table 1), although higher absolute concentrations of the latter inhibitor were required to see elevated 5-BP incorporation, presumably due to the weaker affinity of this acylideneoxindole for TG2 (data not shown). These unexpected results motivated us to reconstitute the dual agonist/antagonist behavior of acylideneoxindoles *in vitro* using recombinant human TG2.

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As shown in Figure 3, at a saturating Ca^{2+} concentration, CK-IV-55 was indeed a non-competitive inhibitor of the V224 variant of TG2 ($K_i = 3.3 \pm 0.9 \mu\text{M}$). However, when catalytic activity of the protein was assessed over a range of sub-saturating Ca^{2+} concentrations in the presence or absence of CK-IV-55, weak but reproducible agonism was observed between 0.5-0.7 mM Ca^{2+} (Figure 4A). These titrations were repeated for each inhibitor listed in Table 1. In all cases, weak but measurable agonism was observed at a Ca^{2+} concentration of 0.6 mM (Figure 4B).

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To assess the effect of CK-IV-55 on the transamidating activity of TG2, a microtiter plate assay was used, where 5-BP is incorporated into immobilized *N-N*-dimethylcasein and quantified using streptavidin²². Guided by the data shown in Figure 4A, a sub-saturating Ca^{2+} concentration of 0.6 mM was used in this assay. Whereas agonism was not observed in the presence of Ca^{2+} alone, CK-IV-55 markedly enhanced the transamidation activity of TG2 in the presence of both 0.6 mM Ca^{2+} and 100 μM GMP-PNP (Figure 4C). Thus, the observed agonistic activity of CK-IV-55 in cell culture (Figure 2) may reflect the presence of some “closed conformation” TG2 in the extracellular matrix of WI-38 fibroblasts, which can be agonized by CK-IV-55 in the presence of sub-saturating (~ 0.5 mM) concentrations of Ca^{2+} . Whereas GTP-bound TG2 has not been reported in the extracellular environment of mammals, the corresponding closed state of the protein is believed to persist due to other (e.g., proteoglycan) interactions²³.

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The non-competitive inhibitory behavior of CK-IV-55 (Figure 3) suggests that acylideneoxindoles are allosteric modulators of TG2. To interrogate the binding mode of these compounds, we first evaluated whether CK-IV-55 induces a conformational change in the protein, analogous to Ca^{2+} and GTP. It is known, for example, that the active site-directed irreversible inhibitor Ac-P(DON)LPF-NH₂, locks TG2 in the open state². However, based on native PAGE analysis, the presence of CK-IV-55 did not have a measurable influence on the relative distribution of the open versus closed states of TG2 (Figure S3). Thus, it is unlikely that acylideneoxindoles bind the enzymatic active site of TG2.

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Structural and mutagenesis studies have shown that the R580 residue contributes significantly to the GTP binding pocket of TG2, as indicated by attenuated GMP-PNP inhibition of the R580A mutant of human TG2^{3,24-26}. As shown in Figure 5, the inhibitory potency of CK-IV-55 was unaltered in this TG2 mutant. Moreover, its agonistic effect in the presence of GMP-PNP was also evident in the R580A mutant. Together, these observations suggest that the binding site of CK-IV-55 is distinct from the GTP-binding pocket of TG2.

The largest of the four domains of human TG2 is its catalytic core; this domain is also the principal site of Ca^{2+} ion binding. To test whether CK-IV-55 targets the catalytic core of

TG2, a truncated form of human TG2 (1-466) lacking both of its C-terminal beta-barrel domains was expressed and purified. This derivative retains deamidating as well as transamidating activity, but lacks the ability to be inhibited by guanine nucleotides^{27,28}. Not only did CK-IV-55 retain its ability to inhibit TG2 (1-466), but it also emerged as a total antagonist of the truncated protein in contrast to its partial inhibitory character on wild-type TG2 (Figure 6). Thus, the binding site of CK-IV-55 lies within the N-terminal domain and/or the catalytic core of TG2.

Taken together, the above findings led us to speculate that CK-IV-55 may exert its unusual agonist/ antagonist effects by interacting with the binding site(s) of one or more Ca^{2+} ions. Although these sites have not been crystallographically defined, detailed biochemical and mutagenesis studies by Király *et al.* have led to a model in which TG2 has one high-affinity ($K_d \sim 1 \mu\text{M}$) and five lower-affinity ($K_d \sim 1 \text{ mM}$) Ca^{2+} binding sites²⁹. Occupancy of the high-affinity binding site (designated S1) and two of the lower affinity binding sites (S2A and S2B) is insufficient for activating TG2; all of the remaining binding sites (S3, S4, and S5) are critical for maximum catalytic activity. We hypothesized that CK-IV-55 bound to one or both of the S2 sites. To test this hypothesis, we introduced the same deleterious mutants at sites S2A and S2B as reported previously by Kiraly, et al. (E396Q/N398S/D400N for S2A and E447Q/E451Q/E452Q/E454Q for S2B)²⁹. As seen in Figure 7, CK-IV-55 had comparable inhibitory effect on the activity of the S2A mutant as the wild-type enzyme, whereas it had no effect on the S2B mutant. Thus, it appears that this inhibitor and, by inference, all other acylideneoxindoles in the series, bind to the S2B Ca^{2+} binding site of the protein.

A simple mechanistic model for the dual agonist/ antagonist characteristics of CK-IV-55 is proposed in Figure 8. According to this model, CK-IV-55 acts as a partial agonist when it binds to the state of TG2 whose low-affinity Ca^{2+} binding sites are unoccupied. In contrast, it is an antagonist when it prevents full occupancy of the Ca^{2+} binding sites of the protein.

In the context of celiac disease, the discovery of a small organic molecule that is capable of partially agonizing the activity of human TG2 provides a fundamentally new insight of potential relevance to disease pathogenesis. It is well established that TG2-catalyzed deamidation of gluten peptides increases their specificity towards disease associated HLA-DQ2 or HLA-DQ8 molecules^{7,8}. However, the mechanism of TG2 activation in the celiac intestine is unknown. The problem is especially vexing as one considers the pathophysiological basis for the breakdown of oral tolerance to dietary gluten, i.e., during the onset of this autoimmune disorder. The prevalence of celiac is on the rise in developed countries³⁰⁻³², and there is growing evidence suggesting a role for environmental triggers other than gluten³³⁻³⁵. Many environmental risk factors have been proposed in recent years based on observational studies, including maternal iron use during pregnancy³⁶, infections with rotavirus³⁷ and *Campylobacter jejuni*³⁸, and exposure to antibiotics³⁹ or to elective Caesarian section⁴⁰; however, in no instance is a mechanistic relationship between the putative cause and effect obvious. Based on our findings in this report, we speculate that small molecules derived from our diet or our microbiota may elevate the activity of extracellular TG2 in the small intestinal mucosa. If so, then chronically elevated TG2 activity in genetically susceptible individuals may enhance the probability of breaking down

oral tolerance to dietary gluten and a concomitant increase in celiac disease prevalence. The discovery of natural and/or man-made products in the human diet or environment that have the ability to mimic the agonistic capacity of CK-IV-55 may promote a better understanding of this major public health concern.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Activation of TG2 by Ca^{2+} . B) Inhibition of TG2 by GMP-PNP at a fixed Ca^{2+} concentration of 5 mM. In all assays the concentration of TG2 was 0.5 μM .

Figure 2.

Activation of extracellular TG2 by CK-IV-55. WI-38 fibroblasts were grown to confluence and pre-incubated with vehicle (1% DMSO) or CK-IV-55 for 30 min. Cells were then incubated with 200 μM 5-BP for 3 h, fixed, and stained. Scale bars = 100 μm. 5-BP incorporation is indicative of TG2 transamidating activity. Images are representative of 5 images sampled across each well.

Figure 3. Inhibition of the deamidation activity of TG2 by CK-IV-55. A) Michaelis-Menten plots; B) Lineweaver-Burk analysis. In all assays $[\text{TG2}] = 0.5 \mu\text{M}$ and $[\text{Ca}^{2+}] = 5 \text{ mM}$.

Figure 4.

A) CK-IV-55 activates TG2 at Ca²⁺ concentrations between 0.5 mM and 0.8 mM; B) Activation of TG2 by various acylideneoxindoles. Inhibitor concentrations used were 3-5x greater than K_i ([CK-IV-55] = 15 μM; [CK-IV-67] = 80 μM; [CK-V-12] = 25 μM; [NMRT3118] = 400 μM). [Ca²⁺] = 0.6 mM; [TG2] = 2.5 μM; [ZQG] = 10 mM; C) Effects of CK-IV-55 on transamidating activity of TG2 in the presence or absence of the stable GTP analog, GMP-PNP; [Ca²⁺] = 0.6 mM.

Figure 5. Deamidation activity of wild-type and R580A TG2 in the presence of CK-IV-55 and GMP-PNP. [TG2] = 0.5 μM; [Ca²⁺] = 3 mM.

Figure 6. Comparison of CK-IV-55 inhibition of wild-type TG2 versus a truncated (1-466) mutant. [TG2] = 0.5 μM; [Ca²⁺] = 5 mM. Enzyme activity was normalized relative to the maximum value of each protein, because the k_{cat} and K_{M} of the two proteins are different.

Figure 7.

A) Comparison of deamidation activity of wild-type (WT) TG2 and a mutant with an inactive S2A binding site. B) Comparison of wild-type (WT) TG2 and a mutant with an inactive S2B binding site. [TG2] = 2.5 μ M; [Ca²⁺] = 5 mM.

Figure 8.

Model for dual agonist/antagonist activity of CK-IV-55. Individual Ca^{2+} binding sites are designated via subscripts, in accordance with the numbering scheme of Király, et al²⁹. (Site 2 binds two Ca^{2+} ions, at positions designated S2A and S2B.) The number of asterisks assigned to a state is approximately proportional to its catalytic activity. States bound to CK-IV-55 are designated with a subscript, i.

Table 1

Inhibition of deamidation activity of TG2 by selected acylideneoxindole inhibitors

Compound	K _i [μM]	Substituents
CK-IV-55	3.3 ± 0.9	R1: 4-Cl; R2: Ph o-OMe; R3: H
CK-IV-67	47 ± 19	R1: 4-Cl; R2: Ph p-Cl; R3: H
CK-V-12	9.1 ± 3.2	R1: 4-Cl; R2: 3'-Pyridyl; R3: Ph
NMRT3118	86 ± 3.5	R1: H; R2: 3'-Pyridyl; R3: H

All inhibitory parameters were determined using the GDH assay¹⁷ ([TG2] = 0.5 μM).