Kinetic Analysis of the Interaction of Tissue Transglutaminase with a Nonpeptidic Slow-Binding Inhibitor

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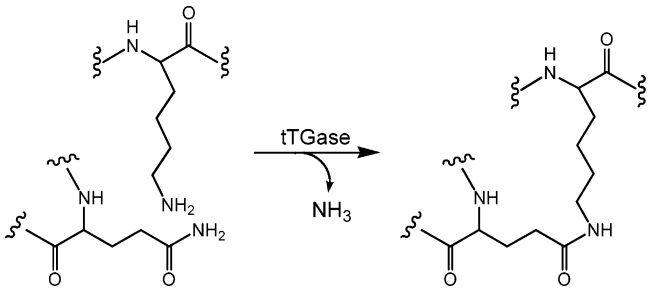
ABSTRACT: Tissue transglutaminase (TGase) is a Ca^{2+}-dependent enzyme that catalyzes cross-linking of intracellular proteins through a mechanism that involves isopeptide bond formation between Gln and Lys residues and is allosterically regulated by GTP. TGase is thought to play a pathogenic role in neurodegenerative diseases by promoting aggregation of disease-specific proteins that accumulate as part of these disorders. Given the role that TGase plays in neurodegenerative disorders, we initiated a research program to discover inhibitors of this enzyme that might ultimately be developed into therapeutic agents. To identify such inhibitors, we screened 110 000 druglike compounds for their ability to inhibit TGase [Case, A., et al. (2005) *Anal. Biochem. 338*, 237−244]. In this paper, we report the kinetics of interaction of human TGase with one of the inhibitors that we identified, LDN-27219. We found that this compound is a reversible, slow-binding inhibitor that appears not to bind at the enzyme's active site but rather at the enzyme's GTP site, or a site that regulates binding of GTP. Interestingly, the potency and kinetics of inhibition are dependent on substrate structure and suggest a novel mechanism of inhibition that involves differential binding of LDN-27219 to multiple conformational states of this enzyme.

Tissue transglutaminase (*1*, *2*) is a Ca^{2+}-dependent enzyme that stably cross-links intracellular proteins through the formation of isopeptide linkages between Gln and Lys residues (see Scheme 1). TGase[1] catalyzes not only reactions of protein substrates but also the transamidation of Gln-containing peptides by a variety of simple primary amine nucleophiles. Principally on the basis of these latter model reactions, a chemical mechanism has emerged for TGase catalysis (*1*) that involves acyl-transfer to and from an active site nucleophile that is thought to be the sulfhydryl moiety of a Cys residue. According to this mechanism, combination of TGase with a Gln-containing substrate to form a Michaelis complex is followed by nucleophilic attack of the active site Cys to generate a covalent acyl−enzyme intermediate and 1 equiv of ammonia. In the presence of a suitable primary amine, the acyl−enzyme intermediate will undergo aminolysis to regenerate free enzyme and the isopeptide product, while in the absence of nucleophile, the acyl−enzyme intermediate reacts with water and undergoes hydrolysis. These mechanistic conclusions, which relate to reactions of simple peptide substrates, have been confirmed in a system in which a protein substrate is used (*3*, *4*).

Scheme 1: Reaction Catalyzed by Tissue Transglutaminase



Significantly, the transamidation activity of TGase is inhibited by GTP and other nucleotides through an allosteric mechanism in which nucleotide binds to a site on the enzyme that is distinct from the active site where acyl transfer chemistry occurs (*5*−*8*). Binding of GTP is thought to induce a conformational change in TGase that weakens the enzyme's interaction with catalytically essential Ca^{2+} cations. Conversely, Ca^{2+} can antagonize the inhibition of TGase by GTP. Bound GTP also undergoes slow hydrolysis to GDP and inorganic phosphate.

Our interest in TGase stems from its likely involvement in the development of a variety of neurodegenerative diseases (*9*−*11*), including Alzheimer's disease (*12*−*15*), Parkinson's disease (*10*, *16*), and Huntington's disease (*17*−*20*). These conditions are often associated with, and are perhaps causally linked to, the accumulation of aggregates of particular proteins. While the in vitro aggregation of these proteins can occur spontaneously, recent results from several laboratories suggest that the in vivo formation of these aggregates

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[1] Abbreviations: TGase, tissue transglutaminase (EC 2.3.2.13); gpTGase, guinea pig enzyme; hTGase, human enzyme; N-Me-casein or NMC, N,N-dimethylated casein; KXD, αN-Boc-Lys-NH-CH_2-CH_2-NH-dansyl; AMC, 7-amido-4-methylcoumarin; $A\beta_{1-40}$, β-amyloid protein peptide comprising residues 1-40, Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val; FI, fluorescence intensity; GDH, glutamate dehydrogenase; αKG, α-ketoglutarate.

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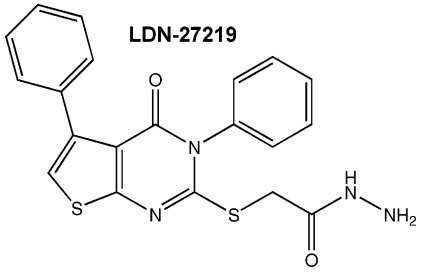
may be facilitated by the cross-linking activity of TGase (*16*, *21*, *22*).

Given the likely involvement of TGase in neurodegenerative disorders, and perhaps other diseases (*23*), this enzyme represents a valid target for inhibitor discovery and design. A number of inhibitors exist for this enzyme, including molecule cystamine (*23*) and several classes of peptide-based irreversible inactivators (*24*−*26*).

To identify inhibitors of this enzyme that might have eventual therapeutic utility in these diseases, we launched a screening program in which we tested the ability of our collection of druglike compounds to inhibit TGase (*4*). The assay used in this screen was based on a detailed kinetic analysis of the enzyme (*3*) and was designed to identify a wide range of inhibitor types (*4*). This screen was successful, and we identified a number of inhibitors. In this paper, we report on one these compounds, LDN-27219 (*27*). We found that LDN-27219 is a reversible, slow-binding inhibitor that appears not to bind at the enzyme's active site but rather at the enzyme's GTP site, or a site that regulates binding of GTP. Interestingly, the potency and kinetics of inhibition are dependent on substrate structure and suggest a novel mechanism of inhibition that involves differential binding of LDN-27219 to multiple conformational states of this enzyme.



MATERIALS AND METHODS

General. Buffer salts, NMC, α-KG, NADH, and GDH were from Sigma Chemical Co. Aβ_{1-40} was prepared by the Biopolymer Lab of Brigham and Women's Hospital (Boston, MA) and stored at −80 °C as 10 mM stock solutions in DMSO. KXD and other peptides were prepared by AbsoluteScience (Cambridge, MA). LDN-27219 and its analogues were from a previous study (*27*). Preparation of gpTGase and hTGase has been described previously (*3*). Unless otherwise noted, the experiments of this study were conducted at 30 °C in a pH 7.4 buffer comprising 50 mM HEPES, 500 mM NaCl, 10 mM $CaCl_2$, and 1 mM DTT.

Kinetic Methods. Kinetic methods for TGase have been described previously (*3*, *4*). Hydrolysis and KXD-promoted transpeptidation of Gln-containing tetrapeptides and Aβ_{1-40} were monitored by following the production of ammonia using a GDH-coupled assay (*3*, *28*).

Factor XIIIa activity was measured in a fluorogenic assay in which enzyme, at a final concentration of 2.7 ng/mL, is added to a pH 8.0 solution (50 mM HEPES, 500 mM NaCl, 10 mM $CaCl_2$, and 5 mM DDT) of 80 μM NMC and 150 μM KXD and the increase in fluorescence at 530 nm (λ_{ex} = 330 nm) monitored. Caspase 3 activity is measured by monitoring the release of AMC (λ_{ex} = 355 nm; λ_{em} = 460 nm) from the peptide substrate Ac-Asp-Glu-Val-Asp-AMC catalyzed by human caspase 3 (10 ng/mL) at pH 7.4 (50 mM HEPES, 150 mM NaCl, 4 mM $MgCl_2$, and 2 mM DDT).

RESULTS

Initial Characterization of LDN-27219. In the course of screening our compound collection for inhibitors of gpTGase (*4*), we identified LDN-27219 as a potent inhibitor of the enzyme, producing 100% inhibition at the screening concentration of 10 μM. In follow-up studies, the compound was shown to inhibit hTGase with an IC_{50} value of 0.6 ± 0.1 μM (n = 3) determined from dose−response curves with concentrations of inhibitor that ranged from 0.1 to 10 μM (*27*).

To examine the enzyme selectivity of LDN-27219, it was tested as an inhibitor of coagulation Factor XIIIa and caspase 3. At concentrations as high as 50 μM, the compound had no effect on the activities of either of these enzymes (data not shown). For these studies, Factor XIIIa was chosen because it is a member of the family of transglutaminase enzymes (*23*) and its inhibition in vivo might lead to compromised coagulation and toxicity. Caspase 3 was chosen because it has a Cys-dependent chemical mechanism for acyl transfer that is essentially identical to TGase's mechanism and it has been implicated in Huntington's disease (*29*). The inhibition of caspase 3 would therefore complicate interpretation of cell-based assays and animal models of efficacy that we plan to conduct with TGase inhibitors.

As part of our routine assessment of validated screening hits, we also examined the cytotoxicity of LDN-27219. The compound was found to be nontoxic at concentrations as high as 100 μM in an assay that measures the viability of HT1080 cells, a fibrosarcoma tumor cell line (data not shown).

Slow-Binding Inhibition of hTGase by LDN-27219. While characterizing the kinetics of inhibition of TGase by LDN-27219, we found that it is a slow-binding inhibitor of both gpTGase and hTGase. In experiments in which enzyme is added to a solution of NMC, KXD, and LDN-27219, the rate of production of product decreases with time until a final, steady-state velocity is reached. An example of a typical reaction progress curve is shown in Figure 1A and can be fit to eq 1, the standard equation for slow binding inhibition (*30*−*32*).

$$[P] = v_{ss}t + \left(\frac{v_o - v_{ss}}{k_{obs}}\right)(1 - e^{-k_{obs}t}) \qquad (1)$$

This equation allows estimation of the initial velocity (v_o), the final steady-state velocity (v_{ss}), and the pseudo-first-order rate constant for the approach to steady state (k_{obs}).

To probe the kinetic mechanism of slow-binding inhibition, we performed a series of experiments in which reaction progress curves were recorded at concentrations of LDN-27219 between 1 and 8 μM while the concentrations of NMC and KXD were held constant at 100 and 20 μM, respectively. As discussed below, while data collected at higher concentrations of inhibitor would have provided more accurate estimates of certain kinetic parameters, attempts to analyze progress curves that were collected at concentrations of inhibitor greater than 8 μM were unsuccessful due to a number of confounding factors, including rapid onset of

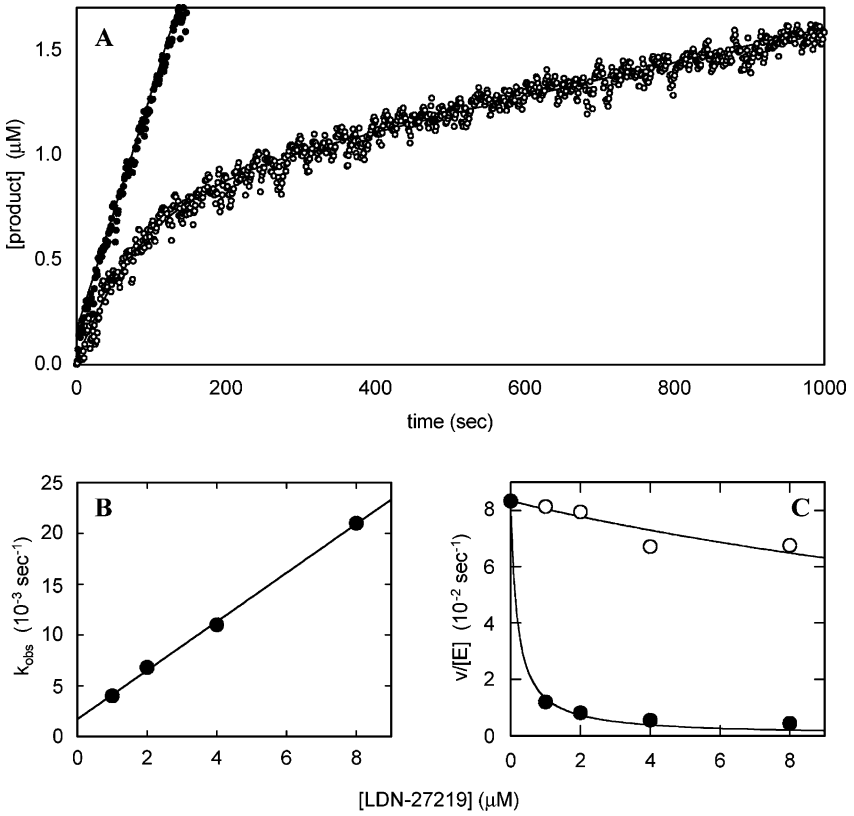


FIGURE 1: Slow-binding inhibition of hTGase by LDN-27219. (A) Reaction progress curves for hTGase-catalyzed transamidation of 100 μM NMC by 20 μM KXD in the absence (●) and presence (○) of 4 μM LDN-27219. The solid line through the control data has a slope of 0.012 μM/s. The solid line through the inhibition progress curve was drawn with the parameters from the best fit of the data to eq 1: $v_o = (10.1 \pm 0.3) \times 10^{-3}$ μM/s, $v_{ss} = (0.86 \pm 0.2) \times 10^{-3}$ μM/s, and $k_{obs} = (11.6 \pm 0.3) \times 10^{-3}$ s^{-1}. (B) Dependence of k_{obs} on inhibitor concentration. The solid line was drawn with the parameters from the best fit of the data to eq 5: $k_{on} = 2400 \pm 58$ M^{-1} s^{-1} and $k_{off} = (1.7 \pm 0.3) \times 10^{-3}$ s^{-1}. (C) Dependence of v_o (●) and v_{ss} (○) on inhibitor concentration. Both data sets were fit to a simple binding isotherm to provide inhibition constants of 28 ± 9 and 0.19 ± 0.03 μM, respectively. The hTGase concentration was 0.15 μM.

inhibition and a decreasing signal-to-noise ratio that occurs as the total amount of product produced decreases at higher inhibitor concentrations.

The resultant progress curves were all analyzed with eq 1. The dependence of k_{obs} on inhibitor concentration is shown in Figure 1B, while the dependencies of v_o and v_{ss} on inhibitor concentration are shown in Figure 1C.

It can be seen that both the initial and steady-state velocities decrease with an increase in LDN-27219 concentration, and in both cases, the dependence of velocity on inhibitor concentration can be described by a simple binding isotherm with apparent inhibition constants of 28 ± 9 and 0.19 ± 0.03 μM for suppression of v_o and v_{ss}, respectively. The larger error associated with the first of these parameter estimates is due to the fact that the highest concentration of inhibitor is only 8 μM, well below any predicted half-sauration point for the dependence of v_o on [I]. These results suggest the mechanism of Scheme 2 in which inhibitor binds rapidly and loosely to enzyme to form $(E{\cdot}I)_1$ which then slowly isomerizes to the more stable $(E{\cdot}I)_2$. The overall dissociation constant for $(E{\cdot}I)_2$ is given by

$$K_i^* = K_i\left(\frac{k_{-i}}{k_i}\right) \tag{2}$$

Note that Scheme 2 depicts a one-substrate reaction. While the same general principles just outlined using this mechanism will be seen to apply to the inhibition of TGase by LDN-27219, the detailed mechanism of inhibition is more complex (see below).

Scheme 2: Mechanistic Scheme for the Slow-Binding, Competitive Inhibitor of a One-Substrate Enzyme

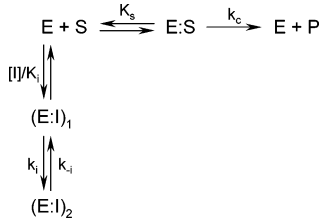


Figure 1B reveals that in the inhibitor concentration range that we employed (i.e., 1−8 μM), the dependence of k_{obs} on LDN-27219 is linear with a slope and intercept of 2.4 ± 0.6 mM^{-1} s^{-1} and $(1.7 \pm 0.3) \times 10^{-3}$ s^{-1}, respectively. At first blush, one would not expect such a linear dependence since, for the mechanism of Scheme 2, k_{obs} has the following dependence on inhibitor concentration:

$$k_{obs} = \frac{k_i[I]}{K_{i,app} + [I]} + k_{-i} \tag{3}$$

where $K_{i,app}$ for the competitive mechanism of this example is equal to $K_i(1 + [S]/K_s)$. Equation 3 describes a hyperbolic, not linear, dependence of k_{obs} on [I]. However, if inhibitor

concentration is low relative to $K_{i,app}$, eq 3 simplifies to the linear function

$$k_{obs} = \frac{k_i}{K_{i,app}}[I] + k_{-i} \quad (4)$$

or

$$k_{obs} = k_{on,app}[I] + k_{off} \quad (5)$$

Given that the highest concentration of inhibitor that we used in the experiments of Figure 1 was 8 μM and that $K_{i,app}$ was estimated to be ~30 μM from the dependence of v_o on [I] (Figure 1C), observing the linear dependence of k_{obs} on [I] is understandable.

Kinetic Mechanism of Inhibition of hTGase by LDN-27219. Having characterized the time dependence of inhibition and, in so doing, established that at least one intermediate species exists along the pathway to the inhibited enzyme, we next set out to determine the kinetic mechanism for the inhibition of hTGase by LDN-27219. Toward this end, we conducted experiments in which we determined values of k_{obs} and steady-state velocities from progress curves that were collected at three inhibitor concentrations (i.e., 0, 0.25, and 0.5 μM) as a function of the concentration of one of the two substrates, while holding constant the concentration of the other substrate. Significantly, we found that while steady-state velocities changed in a systematic way with substrate concentration (see below), values of k_{obs} were independent of substrate concentration.

The steady-state velocity data from these experiments are plotted in Figure 2. In Figure 2A, [KXD] was held constant at 64 μM while [NMC] was varied from 1 to 16 μM, while in Figure 2B, [NMC] was held constant at 100 μM while [KXD] was varied from 8 to 128 μM. Each of the dependencies of v_{ss} on substrate concentration was fit to the Michaelis−Menten equation. The best-fit parameters are summarized in the legend of Figure 2 and plotted in Figure 3 as a function of the inhibitor concentration at which they were determined.

A mechanism-independent analysis can be conducted in which the inhibitor concentration dependencies of $(k_c)_{NMC,obs}$ and $(k_c/K_m)_{NMC,obs}$ (Figure 3A) and $(k_c)_{KXD,obs}$ and $(k_c/K_m)_{KXD,obs}$ (Figure 3B) are fit a to a simple binding isotherm. The apparent inhibition constants derived from this analysis are similar in magnitude (see Figure 3), with an average value of 0.2 ± 0.1 μM. Observation of the same value of $K_{i,app}$ from the dependencies of k_c and k_c/K_m on inhibitor concentration defines noncompetitive inhibition.

A more detailed analysis, informed by what we know about the enzyme's mechanism, can also be attempted. The data we generated to elucidate the mechanism of inhibition of hTGase by LDN-27219 (see Figures 2 and 3) should be interpreted in the context of the kinetic mechanism of Scheme 3 (*3*, *4*). According to this mechanism, TGase first reacts with Gln-donating substrate S to form a stable acyl−enzyme intermediate which then undergoes transamidation by an amine nucleophile N to form S-N. In addition to transamidation, the acyl−enzyme intermediate is also subject to a unimolecular decomposition to form species S′. This unimolecular reaction can potentially be simple hydrolysis or intramolecular transamidiation, or the sum of both. It should

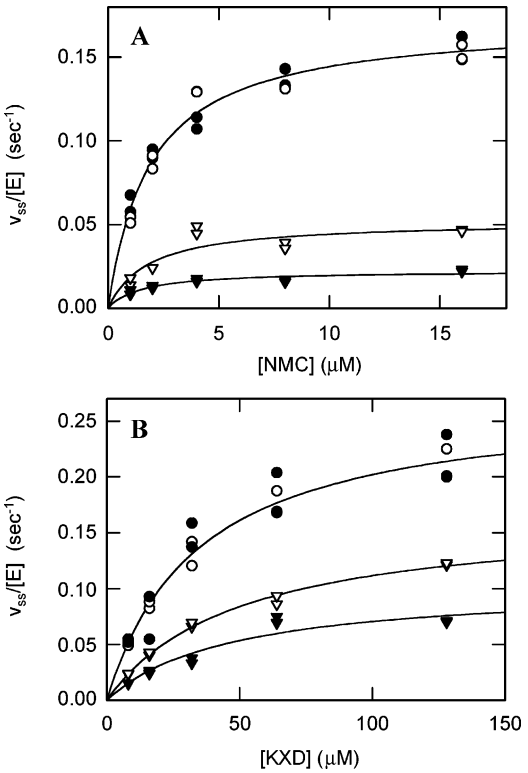


FIGURE 2: Substrate concentration dependencies of steady-state velocities for the inhibition of hTGase by LDN-27219. (A) Dependence of v_{ss} on [NMC] at 64 μM KXD. The concentrations of LDN-27219 that were used and best-fit parameters to the Michaelis−Menten equation are as follows: for 0 μM (○ and ●; two independent experiments), $(k_c)_{NMC,obs} = 0.172 \pm 0.008$ s^{-1} and $(K_m)_{NMC,obs} = 1.8 \pm 0.2$ μM; for 0.25 μM (▽), $(k_c)_{NMC,obs} = 0.052 \pm 0.006$ s^{-1} and $(K_m)_{NMC,obs} = 1.8 \pm 0.7$ μM; and for 0.5 μM (▼), $(k_c)_{NMC,obs} = 0.023 \pm 0.002$ s^{-1} and $(K_m)_{NMC,obs} = 1.5 \pm 0.4$ μM. (B) Dependence of v_{ss} on [KXD] at 100 μM NMC. The concentrations of LDN-27219 that were used and best-fit parameters to the Michaelis−Menten equation are as follows: for 0 μM (○ and ●; two independent experiments), $(k_c)_{KXD,obs} = 0.272 \pm 0.007$ s^{-1} and $(K_m)_{KXD,obs} = 34 \pm 4$ μM; for 0.25 μM (▽), $(k_c)_{KXD,obs} = 0.166 \pm 0.007$ s^{-1} and $(K_m)_{KXD,obs} = 49 \pm 4$ μM; and for 0.5 μM (▼), $(k_c)_{KXD,obs} = 0.103 \pm 0.009$ s^{-1} and $(K_m)_{KXD,obs} = 46 \pm 15$ μM.

be noted that while hydrolysis of Gln residues of peptides and proteins by TGase has been amply demonstrated, intramolecular transamidation remains an unobserved, but formally possible, reaction. In the current studies, S and N correspond to NMC and KXD, respectively.

The rate expression that describes the mechanism of Scheme 3 is given in eq 6

$$\frac{v_{ss}}{[E]} = \frac{k_E[S]\left(\frac{k_{na}[N]}{K_{na} + [N]}\right)}{k_E[S] + k_u^* + \frac{k_{na}[N]}{K_{na} + [N]}} \quad (6)$$

where

$$k_u^* = \frac{k_u}{1 + [N]/K_{na}} \quad (7)$$

k_E is an abbreviation for k_c/K_m for reaction of TGase with S and k_u is the sum of rate constants for hydrolysis and intramolecular transamidation of the acyl−enzyme interme-

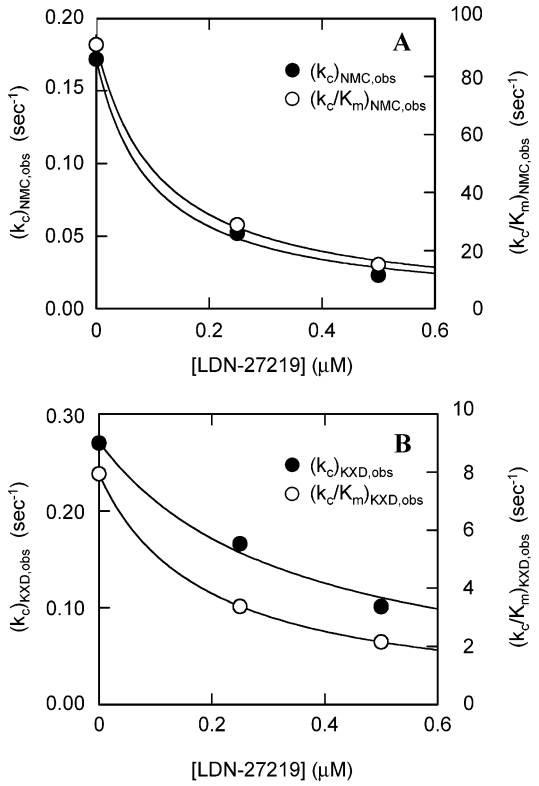


FIGURE 3: Dependence of steady state kinetic parameters on the concentration of LDN-27219. (A) From Figure 2A, values of $(k_c)_{NMC,obs}$ and $(k_c/K_m)_{NMC,obs}$ were plotted as a function of the LDN-27219 concentration at which they were determined and fit to a simple binding isotherm to arrive at the best-fit inhibition constants of 0.10 ± 0.02 mM for the titration of $(k_c)_{NMC,obs}$ by inhibitor and 0.11 ± 0.01 for the titration of $(k_c/K_m)_{NMC,obs}$ by inhibitor. (B) From Figure 2B, values of $(k_c)_{KXD,obs}$ and $(k_c/K_m)_{KXD,obs}$ were plotted as a function of the LDN-27219 concentration at which they were determined and fit to a simple binding isotherms to arrive at the best-fit inhibition constants of 0.32 ± 0.05 mM for the titration of $(k_c)_{KXD,obs}$ by inhibitor and 0.19 ± 0.01 for the titration of $(k_c/K_m)_{KXD,obs}$ by inhibitor.

diate. From previous work (*4*), we know that $k_E = 65\,000$ M^{-1} s^{-1}, $k_u = 0.095$ s^{-1}, $K_{na} = 15$ μM, and $k_{na} = 0.34$ s^{-1}.

When steady-state velocities are measured as a function [NMC] at a constant [KXD], as in Figure 2A, the observed steady-state kinetic parameter are

$$(k_c)_{NMC,obs} = \frac{k_{na}[KXD]}{K_{na} + [KXD]} \quad (8)$$

$$(k_c/K_m)_{NMC,obs} = \frac{k_E}{1 + \frac{k_u}{k_{na}} \times \frac{K_{na}}{[KXD]}} \quad (9)$$

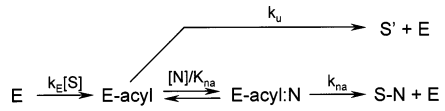
Under the conditions used for Figure 2A where [KXD] was held constant at 64 μM, eqs 8 and 9 simplify to These

$$(k_c)_{NMC,obs} \approx k_{na} \quad (10)$$

$$(k_c/K_m)_{NMC,obs} \approx k_E \quad (11)$$

simplifications allow interpretation of Figure 3A where we now see that the dependence $(k_c)_{NMC,obs}$ on [LDN-27219] reflects the effect of inhibitor predominately on k_{na} and thus

Scheme 3: Mechanism for TGase-Catalyzed Transamidation of Protein Substrate S by Nucleophile N



binding of inhibitor to the E-acyl·N complex and that the dependence $(k_c/K_m)_{NMC,obs}$ on [LDN-27219] reflects the effect of inhibitor predominately on k_E and thus binding of inhibitor to free enzyme E. Dissociation constants for release of inhibitor from E-acyl·N·I and E·I complexes are identical and equal 0.1 μM.

A similar analysis can be carried out for the situation in Figure 2B, where steady-state velocities are measured as a function [KXD] at a constant [NMC]. Here the observed steady-state kinetic parameter are

$$(k_c)_{KXD,obs} = \frac{k_{na}}{1 + \frac{K_{m,n}}{[NMC]}} \quad (12)$$

$$(k_c/K_m)_{KXD,obs} = \frac{k_{na}/K_{na}}{1 + \frac{K_{m,u}}{[NMC]}} \quad (13)$$

where

$$K_{m,n} = \frac{k_{na}}{k_E} \quad (14)$$

and

$$K_{m,u} = \frac{k_u}{k_E} \quad (15)$$

Under the conditions used for Figure 2B where $K_{m,n} = 5$ μM, $K_{m,w} = 1$ μM, and [NCM] was held constant at 100 μM, eqs 12 and 13 simplify to

$$(k_c)_{KXD,obs} \approx k_{na} \quad (16)$$

$$(k_c/K_m)_{KXD,obs} \approx \frac{k_{na}}{K_{na}} \quad (17)$$

Again, these simplifications allow interpretation of the data of Figure 3B where we now see that the dependence $(k_c)_{KXD,obs}$ on [LDN-27219] reflects the effect of inhibitor predominately on k_{na} and thus binding of inhibitor to the E-acyl·N complex and that the dependence $(k_c/K_m)_{KXD,obs}$ on [LDN-27219] reflects the effect of inhibitor predominately on k_{na}/K_{na} and thus binding of inhibitor to the acyl−enzyme intermediate (E-acyl). Dissociation constants for the release of inhibitor from E-acyl·N·I and E-acyl·I complexes are essentially identical and equal 0.3 and 0.2 μM, respectively.

From this analysis and the data depicted in Figure 3, we see that the dissociation constants for the complexes of inhibitor with E, E-acyl, and E-acyl·N are similar in magnitude and equal an average value of ~0.2 ± 0.1 μM. This suggests a classical noncompetitive mechanism of inhibition in which inhibitor binds with approximately the

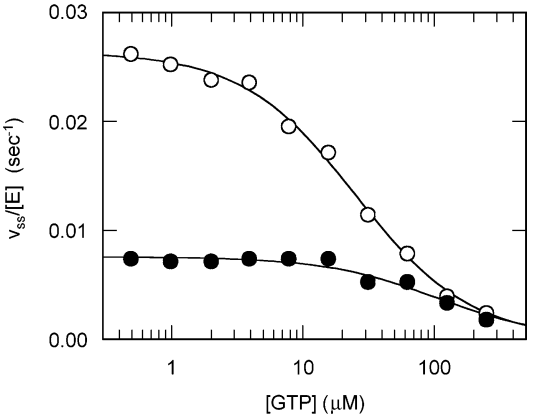


FIGURE 4: Influence of LDN-27219 on the inhibition of hTGase by GTP. At 0 (○) and 0.6 μM LDN-27219 (●), steady-state velocities were determined as a function of GTP concentration for the hTGase-catalyzed transamidation of 8 μM NMC by 16 μM KXD. A global fitting of this to eq 16 yields best-fit parameters ($v_{control} = 0.0263 \pm 0.0003$ s^{-1}, $K_{i,GTP} = 26 \pm 1$ μM, and $K_{i,LDN} = 0.23 \pm 0.01$ μM), which were used, together with eq 16, to draw the solid lines through the data points.

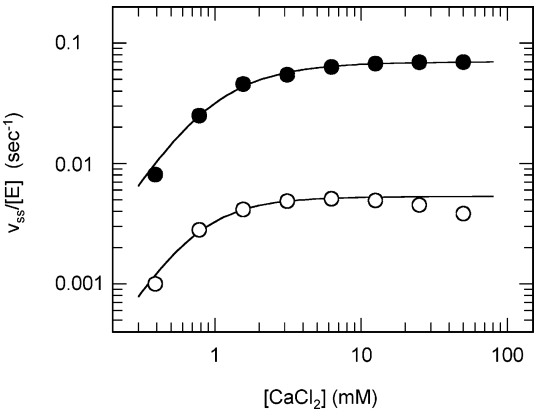


FIGURE 5: Influence of LDN-27219 on the activation of hTGase by calcium. At 0 (○) and 1 μM LDN-27219 (●), steady-state velocities were determined as a function of $CaCl_2$ concentration for the hTGase-catalyzed transamidation of 8 μM NMC by 16 μM KXD. See the text for details of the analysis.

same affinity to the three forms of enzyme that accumulate in the steady state.

Influence of LDN-27219 on the Inhibition of hTGase by GTP. It was of some interest to see if LDN-27219 could modulate the inhibitory effect of GTP, an allosteric inhibitor of TGase, and, if it did, whether it and GTP are mutually exclusive inhibitors of TGase.

To explore this, we needed to study the inhibitory effect that a combination of GTP and LDN-27219 has on TGase. In Figure 4 are the results of such an experiment in which we determined steady-state velocities as a function of [GTP] at two fixed concentrations of LDN-27219 (i.e., 0 and 0.6 μM). Both data sets can be fit to the expression for a simple binding isotherm and yield the following best fit parameters: in the absence of LDN-27219, $(v_{control})_{obs}/[E] = 0.028 \pm 0.001$ s^{-1} and $(K_{i,GTP})_{app} = 25 \pm 1$ μM, and with 0.6 μM LDN-27219, $(v_{control})_{obs}/[E] = 0.0071 \pm 0.0002$ s^{-1} and $(K_{i,GTP})_{app} = 104 \pm 18$ μM.

The observed increase in $(K_{i,GTP})_{app}$ that occurs in the presence of LDN-27219 is consistent with a kinetic mechanism in which the GTP and LDN-27219 are mutually exclusive. The rate expression for this situation is shown in eq 18

$$v_{ss} = \frac{(v_{control})_{obs}}{1 + \dfrac{[GTP]}{(K_{i,GTP})_{app}}} \qquad (18)$$

where

$$(v_{control})_{obs} = \frac{v_{control}}{1 + \dfrac{[LDN\text{-}27219]}{(K_{i,LDN})_{app}}} \qquad (19)$$

and

$$(K_{i,GTP})_{app} = K_{i,GTP}\left[1 + \frac{[LDN\text{-}27219]}{(K_{i,LDN})_{app}}\right] \qquad (20)$$

A global fitting of the data of Figure 4 to eq 18 yields best fit parameters: $v_{control} = 0.0263 \pm 0.0003$ s^{-1}, $K_{i,GTP} = 26 \pm 1$ μM, and $K_{i,LDN} = 0.23 \pm 0.01$ μM. These parameters and eq 18 were used to draw the solid lines through the data points in Figure 4. The excellent fit of the data to the model and the agreement between the value of $K_{i,LDN}$ determined here and the value of 0.2 μM determined in independent experiments (see above) indicate that GTP and LDN-27219 are mutually exclusive inhibitors.

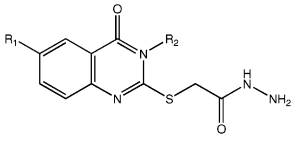
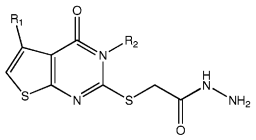
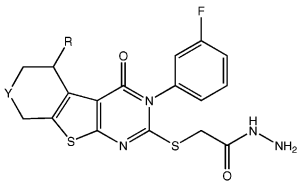
While these data suggest that GTP and LDN-27219 can bind to the same site on TGase, this need not be the case. The binding of GTP occurs at a location that lies between the catalytic core and a region known as the first β-barrel (*8*) and is regulated by the C-terminal region, the second β-barrel (*33*), as well as by the binding of calcium cations (*34*). Thus, it is conceivable that LDN-27219 could bind at sites other than the GTP site and still be mutually exclusive with nucleotide.

Influence of LDN-27219 on the Activation of hTGase by Calcium. TGase has two to six Ca^{2+}-binding sites whose occupancy by the cation is required for the enzyme to express its activity. GTP is thought to inhibit TGase through an allosteric mechanism in which binding of GTP decreases the affinity of the enzyme for Ca^{2+} (*5*, *7*). To explore the possibility that LDN-27219 inhibits TGase by the same mechanism, we conducted an experiment in which we determined steady-state velocities as a function of [$CaCl_2$] at two fixed concentrations of LDN-27219 (i.e., 0 and 1 μM). The results of these experiments are shown in Figure 5 and can be fit to an expression of enzyme activation that requires binding 2 equiv of activator to achieve full activity:

$$v_{ss} = \frac{(v_{control})_{obs}}{1 + K_{1,A}/[A] + (K_{1,A}/[A])(K_{2,A}/[A])} \qquad (21)$$

When [LDN-27219] = 0, $(v_{control})_{obs} = (70 \pm 1) \times 10^{-3}$ s^{-1}, $K_{1,A} = 0.49 \pm 0.16$ mM, and $K_{2,A} = 1.5 \pm 0.8$ mM, and

Table 1: Inhibition of Human Transglutaminase by LDN-27219 and Analogues

Structure			$K_{i,app}$ (μM)		
			transpeptidation NMC by KXD	hydrolysis Z-Pro-Gln-Nle-Phe	transpeptidation $Aβ_{1-40}$ by KXD
					
1	R_1 = H	R_2 = Ph	6	500	-
2	R_1 = Ph	R_2 = 3-F-Ph	1.5	180	-
					
3	R_1 = H	R_2 = Ph	0.25	7.3	39
4	R_1 = Ph	R_2 = 2-F-Ph	0.16	8.2	45
5	R_1 = Ph	R_2 = 3-F-Ph	0.18	10	41
6	R_1=2-OMe-Ph	R_2 = 3-F-Ph	0.14	4.2	19
7	R_1 = 2-($OC_3H_6NEt_2$)Ph	R_2 = 3-F-Ph	0.61	50	210
					
8	Y = CH_2	R = Ph	0.17	10	5
9	Y = N-Boc	R = H	1.1	200	90
10	Y = NH	R = H	7.1	220	110
11	Y = CH_2	R = CH_2-Ph	1.1	190	-

when [LDN-27219] = 1 μM, $(v_{control})_{obs}$ = (5.3 ± 0.1) × 10^{-3} s^{-1}, $K_{1,A}$ = 0.15 ± 0.3 mM, and $K_{2,A}$ = 3.2 ± 0.7 mM.

The values of $(v_{control})_{obs}$ of 0.070 and 0.0053 s^{-1} that were determined at inhibitor concentrations of 0 and 1 μM, respectively, allow the calculation of a $K_{i,app}$ value of 0.08 μM using the simple relationship $K_{i,app} = [I]/(v_c/v_i - 1)$. This value is consistent with the average value of 0.2 ± 0.1 μM determined above.

The results of this experiment also suggest that in contrast to GTP, whose binding prohibits binding of Ca^{2+} (*5*, *7*), LDN-27219 has an only modest influence on the binding of Ca^{2+}, increasing the affinity of the first equivalent of Ca^{2+} by a factor of 3 while reducing the affinity of the second equivalent of Ca^{2+} by a factor of 2.

Inhibition by LDN-27219 and Analogues of the Action of hTGase on Aβ1–40. Given the novel mechanism of inhibition of hTGase by LDN-27219, it was of course of some interest to see if this same mechanism holds with substrates that are important in neurodegeneration. To this end, we examined the effect of LDN-27219 on the hydrolysis and KXD-promoted transamidation of the single Gln residue of $Aβ_{1-40}$.

In the absence of inhibitor, and using kinetic methods that we have previously described (*3*, *4*), we determined the following kinetic parameters for hTGase-catalyzed transamidation of $Aβ_{1-40}$ by KXD (see Scheme 1 for parameter definitions): k_E = 660 M^{-1} s^{-1}, k_u = 0.015 s^{-1}, K_{na} = 44 μM, and k_{na} = 0.11 s^{-1} (data not shown). To assess the potency of LDN-27219 and several of its structural analogues, we conducted inhibitor titration experiments in which inhibitor concentration dependencies of initial velocities were determined after a 30 min preincubation of enzyme and inhibitor, at $Aβ_{1-40}$ and KXD concentrations of 50 and 10 μM, respectively. These data are summarized in Table 1, and a plot of the titration data for LDN-27219 (compound **3** of Table 1) is shown in Figure 6. Also in this figure are the data for a titration experiment in which NMC is used as substrate. To our surprise, these compounds are much weaker inhibitors of the action of hTGase on $Aβ_{1-40}$ than of the action on NMC.

In related experiments, we found that the simple hydrolysis of the Gln of $Aβ_{1-40}$ is also much less sensitive to the inhibitors than is the hydrolysis of Gln residues of NMC. For example, $K_{i,app}$ values for inhibition by LDN-27219 of NMC and $Aβ_{1-40}$ hydrolyses are 0.39 and 26 μM, respectively (data not shown).

Inhibition by LDN-27219 and Analogues of the hTGase-Catalyzed Hydrolysis of Z-Pro-Gln-Nle-Phe. Given the propensity of $Aβ_{1-40}$ to form oligomers, we decided to examine the effect of our inhibitors on simpler peptides in our attempts to understand the apparent substrate structural dependence of inhibitory potency for this series of com-

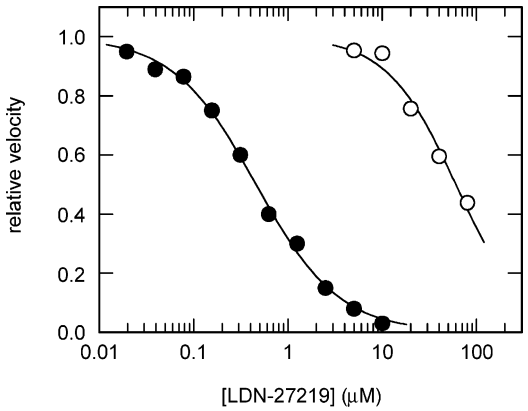


FIGURE 6: Inhibition by LDN-27219 of the hTGase-catalyzed transamidation of NMC (●) and Aβ_{1-40} (○). After a 30 min preincubation of 50 nM hTGase and the indicated concentration of LDN-27219, either 8 μM NMC and 16 μM KXD or 50 μM Aβ_{1-40} and 10 μM KXD were added to initiate the reaction. Initial velocities were recorded and are expressed here relative to the velocity in the absence of inhibitor. Both curves can be fit to a simple binding isotherm with best-fit inhibition constants of 0.46 ± 0.02 and 60 ± 5 μM for transamidation of NMC and Aβ_{1-40}, respectively.

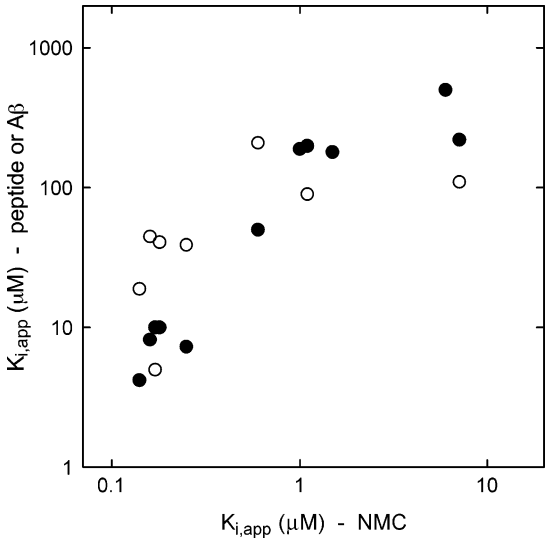


FIGURE 7: Correlation of $K_{i,app}$ values for the inhibition by LDN-27219 and its analogues of hTGase-catalyzed transpeptidation of NMC by KXD with a $K_{i,app}$ for hydrolysis of Z-Pro-Gln-Nle-Phe (●) and a $K_{i,app}$ for transpeptidation of Aβ_{1-40} by KXD (○).

pounds. The substrates we used in these studies were chosen from our investigations of the substrate selectivity of hTGase toward Gln-containing tetrapeptides of the general structure Z-Pro-Gln-Xaa-Xaa.[2] In the first of these studies, we characterized the inhibition of the hTGase-catalyzed hydrolysis of Z-Pro-Gln-Nle-Phe ($k_c = 0.11$ s^{-1}; $K_m = 0.35$ mM). Values of $K_{i,app}$ for LDN-27219 and several of its structural analogues are summarized in Table 1 and presented in Figure 1 as a correlation with $K_{i,app}$ values for inhibition of the hTGase-catalyzed transpeptidation of NMC by KXD. Also included in this figure is a similar correlation with $K_{i,app}$ values for transpeptidation of Aβ by KXD. From this figure, it is clear that inhibition of hTGase-catalyzed reactions of

[2] These studies on the substrate selectivity of TGase will form the basis of a manuscript by us and Dr. Alan Jacobson (Absolute Science, Inc., Cambridge, MA).

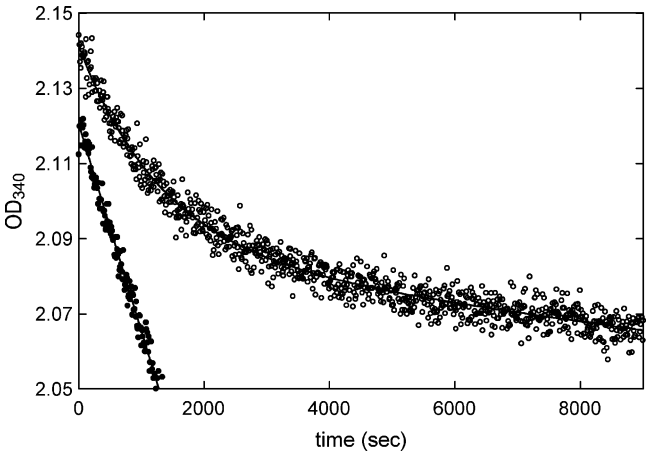


FIGURE 8: Slow-binding inhibition by LDN-27219 of the hTGase-catalyzed hydrolysis of Z-Gln-Pro-Gly-Trp, where [LDN-27219] = 20 μM, [Z-Gln-Pro-Gly-Trp] = 630 μM, and [hTGase] = 0.15 μM. The solid line through the control data has a slope of 5.5×10^{-5} OD/s. The solid line through the inhibition progress curve was drawn with the parameters from a best fit of the data to eq 1: $v_o = (4.4 \pm 0.1) \times 10^{-5}$ OD/s, $v_{ss} = (0.23 \pm 0.07) \times 10^{-5}$ OD/s, and $k_{obs} = (0.75 \pm 0.03) \times 10^{-3}$ s^{-1}.

NMC is much more pronounced than for reaction of either Z-Pro-Gln-Nle-Phe or Aβ.

Given these results, we next wanted to determine the kinetic mechanism of inhibition of LDN-27219 using a tetrapeptide as a substrate. For this study, we chose to use Z-Pro-Gln-Ala-Trp as a substrate ($k_c = 0.13$ s^{-1}; $K_m = 1.6$ mM) because of its relatively high K_m value, which allowed us to precisely measure reaction velocities even at substrate concentrations below K_m. In these experiments, we preincubated the enzyme and various concentrations of inhibitor for 30 min and then added substrate to initiate the reaction. From the dependence of the initial velocity on inhibitor concentration, we determined $K_{i,app}$ values. We observed a positive dependence of $K_{i,app}$ on substrate concentration; that is, at substrate concentrations of 0.5, 1.5, and 4 mM, we determined $K_{i,app}$ values of 3.5, 25, and 110 μM, respectively. These data indicate that unlike the noncompetitive inhibition of reactions of hTGase with NMC, LDN-27219 is a competitive inhibitor of the hTGase-catalyzed hydrolysis of Z-Pro-Gln-Ala-Trp. However, it is obvious that the dependence of $K_{i,app}$ on substrate concentration is extreme, suggesting a mechanism other than simple competitive inhibition.[3] A likely explanation for the unusual dependence of $K_{i,app}$ on $[S]_o$ is that LDN-27219 is a slow-binding inhibitor of the hydrolysis of this substrate, in which case the approach to steady state would proceed at a slower rate at higher substrate concentrations thus giving inflated values of $K_{i,app}$ at these substrate concentrations.

To test this idea, we followed the time course for inhibition by LDN-27219 of the hTGase-catalyzed hydrolysis of Z-Gln-Pro-Ala-Trp. A typical time course is shown in Figure 8 and, when compared to the data of Figure 1, indicates an onset of inhibition that is 2 orders of magnitude slower than that observed during inhibition of the action of hTGase on NMC; that is, from the curve of Figure 8, we calculate a

[3] For a simple competitive inhibitor, $K_{i,app}$ will increase linearly with $[S]_o$ according to an equation of the general form $K_{i,app} = K_i(1 + [S]_o/K_m)$. For this reaction, $K_{i,app}$ depends on $[S]_o$ in a nonlinear, high-order manner.

k_{obs}/[I] value of 20 M^{-1} s^{-1} which should be compared to a k_{on} value of 2400 M^{-1} s^{-1} for the latter reaction. From the final steady-state velocity of curve of Figure 8, a $K_{i,app}$ value of 0.8 μM can be calculated from the expression $K_{i,app} = [I]_o/(1 + v_{control}/v_{ss})$.

DISCUSSION

Our program to discover inhibitors of hTGase identified LDN-27219 as a relatively potent inhibitor of this enzyme. Encouraged by favorable results in enzyme selectivity and cytotoxicity assays, we simultaneously launched a medicinal chemistry program to optimize the potency and biopharmaceutical properties of LDN-27219 (*27*) and began a series of studies aimed at elucidating the mechanism by which this compound inhibits hTGase. The latter work is the subject of this paper.

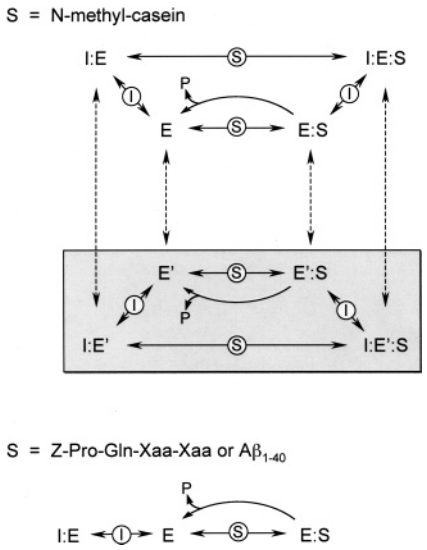
Our kinetic analysis of the inhibition of hTGase by LDN-27219 reveals a number of intriguing, substrate-dependent features that point to a unique mechanism of inhibition. First, for reactions of NMC, we observe the following: (i) slow approach to steady state ($k_{on} \sim 10^3$ M^{-1} s^{-1}), (ii) noncompetitive inhibition with both acyl-donating substrate NMC and nucleophile KXD, and (iii) exclusive inhibition with GTP. In contrast, for reactions of peptidic substrates, we observe (i) a much slower approach to steady state ($k_{on} \sim$ 10 M^{-1} s^{-1}) and (ii) competitive inhibition. The fundamental question we have to address is how substrate structure dictates inhibitor mechanism.

The starting point for formulating an answer to this question is to consider the kinetic consequences that can accompany allosteric inhibition. Recall that our data suggest that LDN-27219 binds to a site on the enzyme that is distinct from the active site and may be the GTP site, or at least at a site that regulates the binding of GTP. Binding of LDN-27219 at an allosteric site can explain our observation of noncompetitive inhibition for reactions of hTGase with NMC. In such a mechanism, binding of inhibitor would induce a conformational change of the enzyme that would not only inhibit binding of the substrate but also shut down enzymatic turnover of the substrate.

A noncompetitive mechanism of inhibition can also be used to explain how the potency of an inhibitor might depend on substrate structure since for this mode of inhibition the inhibitor binds not only to the free enzyme but also to a form of the enzyme in which substrate is present, that is, the E·S complex. While binding of inhibitor to free enzyme clearly cannot be dependent on substate structure, binding of inhibitor to the E·S complex can be sensitive to substrate structure. For example, one can imagine a situation in which for S_1, inhibitor can bind to E·S_1 to form I·E·S_1, while for structurally distinct S_2, inhibitor cannot bind to E·S_2 and the I·E·S_2 complex does not form. In this case, noncompetitive inhibition would be seen for reaction of S_1 but competitive inhibition would be observed for S_2.

We believe that this, at least in part, explains the situation with LDN-27219, since we observe noncompetitive inhibition with NMC but competitive inhibition with peptidic substrates. However, there must be an additional mechanistic feature at work since we also observe that the kinetics of binding of LDN-27219 to hTGase is much slower when substrate is peptide rather than NMC (i.e., k_{obs}/[I] = 20 and 2400 M^{-1} s^{-1} for binding of peptide and NMC, respectively). This cannot be explained by the mechanism we just outlined since the very slow approach to steady state that we observe for peptide corresponds to binding of inhibitor to free enzyme, and the rapid binding of inhibitor observed when substrate is NMC occurs with all enzyme forms, including free enzyme. The only way to explain this is to posit a mechanism in which hTGase can exist in at least two catalytically active conformations. This mechanism is illustrated in Scheme 4, where, for the sake of clarity, hTGase is shown as a one-substrate enzyme and binding of inhibitor is shown to occur in one step, rather than two.

Scheme 4: Substrate-Dependent Inhibition of hTGase by LDN-27219[a]



[a] Double-headed arrows represent reversible interconversion, and the circled S and I indicate that the interconversion is the equilibrium addition of substrate and inhibitor, respectively. The shaded area in the top mechanism where S = *N*-methylcasein corresponds to the manifold of reactions mediated by enzyme form E′.

According to this mechanism, when substrate is NMC (i) enzyme exists in conformational states E and E′, and E·S and E′·S, (ii) E·S and E′·S are catalytically competent, (iii) E and E·S, as well as E′ and E′·S, can bind LDN-27219, (iv) all conformational isomerizations are rapid relative to catalysis, (v) "primed" enzyme forms, which are set off in the gray box, are thermodynamically favored, and (vi) inhibitor binds much slower to E and to E·S than it does to either E′ or E′·S. In contrast, when substrate is peptide (i) LDN-27219 binds to only E and not to E·S, (ii) peptide substrates cannot bind to E′, and (iii) E′·S does not form when S is peptide.

Given the mechanism of Scheme 4 and these substrate-dependent features, we can explain our observations in the following way. We see competitive, very slow-binding inhibition when substrate is peptide, because LDN-27219 is able to bind only to E, and to this form quite slowly. In contrast, we see noncompetitive and a more rapid (but still slow) attainment of full inhibition when substrate is NMC, because inhibitor can now bind to all forms of enzyme and binding of inhibitor to E′ and E′·S is faster than binding to E and E·S. Since E′ and E′·S predominate, binding of inhibitor is more rapid than if binding was to E and E·S.

Summary and Conclusion. In a screening campaign to discover inhibitors of TGase, we identified LDN-27219, a heterocyclic compound with an IC_{50} value slightly less than 1 μM. We found that LDN-27219 is a slow-binding inhibitor of TGase, with potency and mechanism dependent on substrate stucture. This report should be viewed not only as the description of a novel inhibitor but also as a cautionary tale to all those engaged in the search for enzyme inhibitors using methods of high-throughput screening, exhorting them to take care in assay development and subsequent inhibitor characterization.

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