Chemistry & Biology
Article

Identification of Chemical Inhibitors to Human Tissue Transglutaminase by Screening Existing Drug Libraries

Thung-S. Lai,[1,*] Yusha Liu,[1] Tim Tucker,[1] Kurt R. Daniel,[2] David C. Sane,[2] Eric Toone,[3] James R. Burke,[1] Warren J. Strittmatter,[1] and Charles S. Greenberg[4,*]
[1]Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA
[2]Department of Internal Medicine-Cardiology, Wake Forest University School of Medicine, Winston-Salem, NC 27157-1045, USA
[3]Department of Chemistry, Duke University, Durham, NC 27708, USA
[4]Department of Pathology, Duke University Medical Center, Durham, NC 27710, USA
*Correspondence: t.lai@duke.edu (T.S.L.), charles.greenberg@duke.edu (C.S.G.)
DOI 10.1016/j.chembiol.2008.07.015

SUMMARY

Human tissue transglutaminase (TGM2) is a calcium-dependent crosslinking enzyme involved in the post-translational modification of intra- and extracellular proteins and implicated in several neurodegenerative diseases. To find specific inhibitors to TGM2, two structurally diverse chemical libraries (LOPAC and Prestwick) were screened. We found that ZM39923, a Janus kinase inhibitor, and its metabolite ZM449829 were the most potent inhibitors with IC_{50} of 10 and 5 nM, respectively. In addition, two other inhibitors, including tyrphostin 47 and vitamin K_3, were found to have an IC_{50} in the micromolar range. These agents used in part a thiol-dependent mechanism to inhibit TGM2, consistent with the activation of TGM2 by reduction of an intramolecular disulfide bond. These inhibitors were tested in a polyglutamine-expressing *Drosophila* model of neurodegeneration and found to improve survival. The TGM2 inhibitors we discovered may serve as valuable lead compounds for the development of orally active TGM2 inhibitors to treat human diseases.

INTRODUCTION

Tissue transglutaminase (TGM2) is a member of the transglutaminase (TG, E.C. 2.3.2.13, protein-glutamine γ-glutamyltransferase) family, known to catalyze inter- and intramolecular crosslinking between specific glutamine γ-carboxamide groups and ε-amino groups in lysine and free primary amines (TGase) (Folk, 1983; Greenberg et al., 1991; Lorand and Graham, 2003). TGM2 is ubiquitously expressed and in almost all tissues (Folk, 1983; Greenberg et al., 1991; Lorand and Graham, 2003), and is found on the cell surface—membrane bound—in the extracellular matrix, cytoplasm, and nucleus of cells (Greenberg et al., 1991; Lorand and Graham, 2003). Extracellular TGM2 interacts with integrins and fibronectin, and is involved in cell movement, adhesion, and proliferation (Lorand and Graham, 2003). Intracellular TGM2 crosslinking activity is inhibited when bound to guanosine-5′-triphosphate (GTP) (Achyuthan and Greenberg, 1987). TGM2 also functions as a G protein in adrenergic receptor-mediated and phospholipase C signal transduction pathways (Murthy et al., 1999; Nakaoka et al., 1994). In addition, TGM2 can bind and hydrolyze ATP, and function as a kinase (Lai et al., 1998; Mishra and Murphy, 2004a). Understanding how TGM2 can perform such diverse functions in cells remains a challenging task, but new studies reveal the protein can exist in two different conformations (Pinkas et al., 2007). When bound to GTP/GDP, the protein adopts a compact protease-resistant conformation. When a substrate interacts with the protein, disulfide bond is formed and the enzyme has an elongated open conformation that exposes the active site (Pinkas et al., 2007). The active site pocket of TGM2 is composed of a catalytic triad of C^{277}-H^{335}-D^{358} (Liu et al., 2002), and the first rapid step in catalysis involves the formation of a transitional thioester bond between active site C^{277} and the Q substrate (Case and Stein, 2003) that requires a specific set of interactions that potentially could be disrupted by a small molecule.

TGM2 is an important therapeutic target for several neurodegenerative diseases, including Huntington's, Alzheimer's, and Parkinson's diseases (Malorni et al., 2008; Muma, 2007). TGM2 crosslinking alters the solubility, structure, and function of proteins that express a polyglutamine repeat (Lai et al., 2004), such as alpha-synuclein (Andringa et al., 2004) and Tau (Tucholski et al., 1999). Neuronal TGM2 contributes to distinctive pathological features of many neurodegenerative diseases mediated by central nervous system (CNS) expression of polyQ protein (Arrasate et al., 2004; Konno et al., 2005). Studies using TGM2 KO mice mated with Huntington's disease (HD-prone mice) showed that TGM2 plays a role in the neurodegenerative progression of polyQ disease (Mastroberardino et al., 2002). Because polyQ disease is a fatal illness that has no known therapy, efforts to treat this disorder are needed. Furthermore, other diseases where there is formation inside and outside the CNS may also benefit from the development of orally active TGM2 inhibitors. Given the involvement of TGM2 in such severe diseases, the development of small-molecule inhibitors capable of inhibiting TGM2 protein crosslinking is warranted. In this study, we used a high-throughput screening assay to determine whether

any existing compounds could inhibit TGM2 protein crosslinking and were active in the CNS when administered orally.

In an effort to identify inhibitors of TGM2, we screened two structurally diverse chemical libraries, including LOPAC (Sigma-Aldrich; St. Louis, MO) and Prestwick (Prestwick Chemical; Illkirch, France). The LOPAC library contains 1,280 pharmacologically active compounds that span a broad range of biological arenas. This library contains marketed drugs, failed development candidates, and "gold standards" that have well-characterized activities. The Prestwick Chemical Library consists of 880 carefully selected compounds, which are highly diverse in structure and cover many therapeutic areas. Our approach to identify inhibitors to a new therapeutic target could significantly shorten the interval between preclinical and clinical studies (Chong and Sullivan, 2007). In this study, we report three chemicals that inhibit TGM2 in vitro and are bioactive in vivo. These inhibitors bound tightly to TGM2, and inhibition was modified by the reducing agent DTT, suggesting a disulfide bridge plays a critical role in their mechanism of action. Our data strongly suggest that these three chemicals could be used as scaffold structures for designing more specific TGM2 inhibitors that are orally active and could be used to treat CNS disorders in which TGM2 plays a role.

RESULTS

Screening of LOPAC and Preswick Chemical Libraries

Initial screening was performed using a continuous solution-phase fluorescent KXD incorporation assay performed at 6 μg/ml of purified recombinant human TGM2 in the presence of 10 mM Ca^{+2} in a 96-well microtiter plate designed for robotic high-throughput screening (see Experimental Procedures). Initially, 20 hits were identified based on inhibition of ≥90% of TGase/TGM2 activity at 50 μM of candidate inhibitor, excluding the well-characterized inhibitors cystamine (CYST) and iodoacetamide, which target the active site Cys-SH of TGM2. These chemicals were more potent than GTP, as 50 μM of GTP inhibited ≤25% of TGase/TGM2 activity under the same conditions. To eliminate fluorescence interference, chemicals were subjected to a secondary screening using a colorimetric BP incorporation assay (see Experimental Procedures). A total of nine inhibitors were found to have IC_{50} ≤ 20 μM (Tables 1 and 2). These inhibitors can be classified as kinase-, phosphatase-, or quinone-related inhibitors (Tables 1 and 2). The kinase- and phosphatase-related inhibitors target kinases and phosphatases, including c-Raf-1 kinase (Ser/Thr kinase), Janus kinase 3 (JNK3, a Tyr kinase), EGFR tyrosine kinase, calmodulin (CAM) kinase, and Cdc25 tyrosine phosphatase. Several of these inhibitors were developed to target GTP (GW5074, tyrphostin 47, Me-3,4-dephostatin) and adenosine-5′-triphosphate (ATP) binding sites with guanine (and adenine) as backbone structures. The naphthoquinone-related TGM2 inhibitors (NSC95397, β-lapachone, and menadione, or vitamin K_3 as is it also known) can be classified into 1,2-naphthoquinone (β-lapachone) or 1,4-naphthoquinone (NSC95397 and vitamin K_3) derivatives (Table 2). Reactive blue 2 is an anthraquinone derivative.

ZM 39923, initially designed to inhibit Janus kinases (Luo and Laaja, 2004), was found to be a potent inhibitor of TGM2 with IC_{50} of 10 nM when the assay was performed in the absence of DTT (Tables 1 and 2). ZM 449829 (Figure 1 and Table 3), the metabolite of ZM 39923 (Luo and Laaja, 2004), was an even more potent inhibitor with an IC_{50} of 5 nM.

Effects of Triton X-100 on Inhibitors in Inhibiting TGase/TGM2 Activity

To eliminate compounds that were nonspecific or low affinity, we investigated the effects of Triton X-100. Small molecules may form nonspecific submicrometer aggregates. A reversible physical association between aggregates and enzyme could produce a false positive in the screening assay (McGovern et al., 2003). Small aggregates are eliminated by 0.01% Triton X-100 treatment (McGovern et al., 2003), and we found the potency of GW 5074, Rotterin, reactive blue 2, and β-lapachone were significantly reduced by 0.02% Triton X-100 treatment (Tables 1 and 2), whereas ZM39923 was not changed. In contrast, the IC_{50} of Me-3,4-dephostatin, tyrphostin 47, NSC95397, and vitamin K_3 were increased by 1.3-fold to 2.5-fold after Triton X-100 treatment (Tables 1 and 2).

Effects of Tyrphostins-Related Compounds on TGM2 Activity

Tyrphostins are a class of synthetic compounds that have been identified as the inhibitors of EGF receptor tyrosine kinase (Wolbring et al., 1994). There is a comprehensive collection of tyrphostin analogs in the LOPAC library (see Tables S1–S4 available online). We identified Me-3,4-dephostatin and tyrphostin 47 as two potent inhibitors in tyrphostin-related compounds with IC_{50} < 20 μM (Table 1).

Effects of Reducing Agent on Inhibition of TGM2 Activity

Because active TGM2 requires free sulfhydryl (SH) groups to be active, we tested the effect of reducing agents on TGM2 inhibition. We found that the inhibition of vitamin K_3, vitamin K_2 (MK4), NSC95397, T7540 (tyrphostin 47), ZM39923, and ZM449828 was reduced by ~23-, 17-, 50-, 17-, 300-, and 4,000-fold, respectively, in the presence of DTT (Table 3).

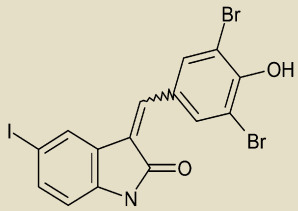
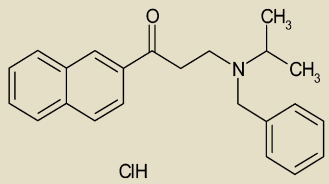
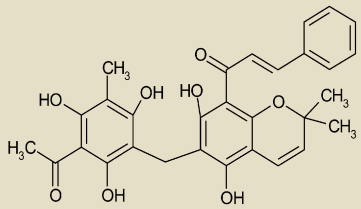
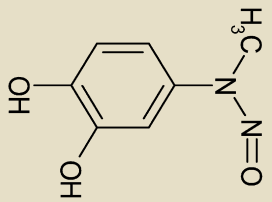
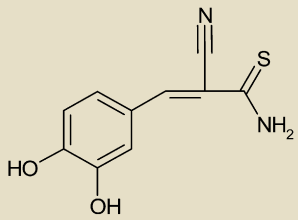
Effects of Naphthoquinone Analogs on Inhibition of TGase Activity

To investigate which functional groups in naphthoquinones were important for inhibition of TGase/TGM2 activity, a series of 1,4-naphthoquinone analogs were investigated at a concentration of 15 μM (Table S5). For the 1,4-naphthoquinone analogs, a methyl group (vitamin K_3) or no substitution (152757) at the C-2 position appeared to be important in inhibiting TGase activity (Table S5). Substitution with other groups reduced the inhibitory potency (Table S5). The side chain at positions 2 and/or 3 in naphthoquinone were also investigated and were less potent, with the exception of MK4, a naturally occurring vitamin K_2 analog with a geranylgeranyl side chain (Table S5). Vitamin K_3 and vitamin K_2 inhibited TGase activity to a similar extent, whereas vitamin K_1 (phylloquinone) did not (Table S5). Ubiquinone (CoQ_{10}), a commonly used antioxidant, was not an inhibitor of TGM2.

Calcium Ions Lead to Irreversible Inhibition of TGM2

To determine whether the inhibitors would produce reversible inhibition, dialysis experiments were performed (Figure 2A). When TGM2 was incubated with inhibitors in the absence of Ca^{+2}, ZM39923, vitamin K_3, vitamin K_2, and Me-3,4-dephostatin

Table 1. Kinase-Related Inhibitors

Name	IC_{50} (μM)	IC_{50} (Triton#)	Target	Structure
G6416 (GW5074)	15 ± 6	50 ± 5	cRaf kinase	
Z4626 (ZM 39923)	0.01 ± 0.01	No Change	Janus Kinase 3	
R5648 (Rottlerin)	20 ± 10	> 50	pKC & CaM III Kinase	
M9440 (Me-3,4-dephostatin)	18 ± 8	36 ± 10	Tyrosine Phosphatase	
T7540 (Tyrphostin 47)	3 ± 3	4 ± 2	EGFR Tyrosine Kinase	

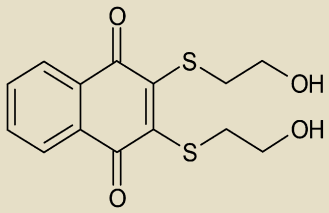
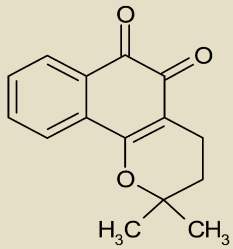
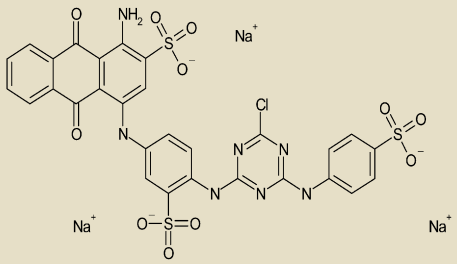
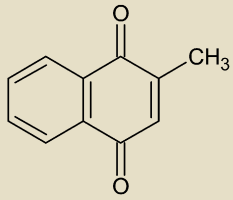
1. The activities were determined by BP-assay in the absence of DTT and represent the average of two triplicate experiments.
2. #: 0.02% Triton was used in the assay.

were reversible inhibitors (Figure 2A). In contrast, TGM2 activity after incubation with ZM449829, tyrphostin 47, and NSC95397 were only partially reversible (Figure 2A). In the presence of Ca^{+2}, the inhibition was irreversible (Figure 2B) in all cases.

Effects of Inhibitors on Crosslinking Activity of Blood Coagulation Factor XIIIa (FXIIIa)

We investigated whether inhibitors could modify the plasma protein FXIIIa, a member of the transglutaminase family (Greenberg et al., 1991; Lorand and Graham, 2003). FXIIIa is formed after thrombin cleavage and release of activation peptides and the dissociation of factor XIII B chains (Lorand and Graham, 2003). ZM39923, Me-3,4-dephostatin, tyrphostin 47, and NSC96397 showed significant inhibition of crosslinking activity with 15 μM of the chemical in the absence of DTT (Table 4). FXIIIa activity was also partially restored by DTT (Table 4). The IC_{50} of the selected inhibitors are presented in Table 4 and are in the same range and pattern of inhibition for TGM2.

Table 2. Quinone-Related Inhibitors

Name	IC_{50} (μM)	IC_{50} (μM) (Triton#)	Target	Structure
N1786 (NSC95397)	1 ± 0.5*	2.5 ± 0.6*	Cdc25 Phosphatase	
L2037 (β-lapachone)	5 ± 3	20 ± 2	Anti-Cancer	
R115 (Reactive Blue 2)	1.8 ± 0.4*	> 50*	P_2Y Receptor	
Menadione (Vitamin K_3)	2.2 ± 0.8*	5.5 ± 2*	ReDox	

*Performed W/O DTT.
#: 0.02% Triton was used in the assay.
1. The activity was determined by BP-incorporation assay and represent the average of two triplicate experiments.

Effects of Inhibitors on Proteolysis of TGM2

GTP binds to TGM2 and renders the molecule protease resistant, whereas ATP binding has less effect. TGM2 was incubated with 100 μM of the inhibitors, and trypsin proteolysis was monitored by SDS-PAGE. (Figure S1). Tyrphostin 47-treated TGM2 was protected from proteolysis in a pattern similar to GTP. All other compounds showed limited proteolytic protection and had patterns similar to ATP (Figure S1).

Effects of Chemical Inhibitors on GTP Binding of TGM2

The GTP binding properties were validated using the fluorescent GTP analog BODIPY-FL-GTP. Binding of this analog to TGM2 produces a dramatic enhancement of fluorescence intensity (Figure 3). GTP and GDP compete with BODIPY-GTP and reduced fluorescent intensity (Figure 3A). Tyrphostin 47 was able to compete with GTP for the binding (Figures 3B and 3C), suggesting that they bind to TGM2 in a similar manner.

Effects of Chemicals That Altered TGM2 Activity on a Drosophila Model of Human polyQ Disease (Machado-Joseph Disease)

Because the long-term goal of our project is to develop orally active agents to inhibit TGM2 for use in neurodegeneration, we tested them in a *Drosophila melanogaster* model of a polyQ

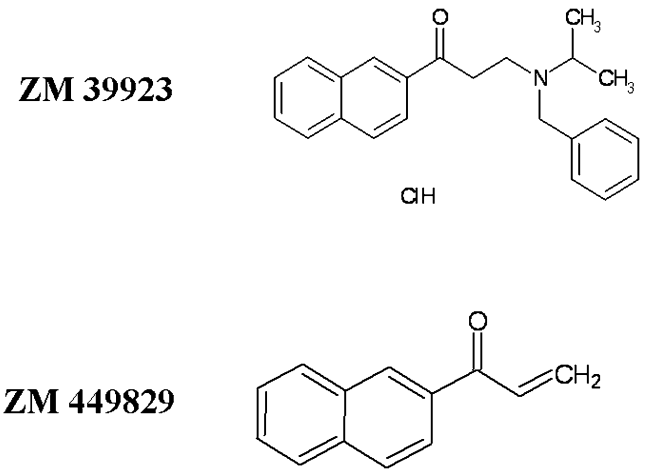


Figure 1. Structures of ZM39923 and ZM449829

repeat disorder called Machado-Joseph disease (MJD) (Figure 4; Arrasate et al., 2004). Expression of the mutant human polyQ disease gene in *Drosophila* confers a phenotype similar to the human disease with late-onset, progressive neural degeneration (Nagai et al., 2007). *Drosophila*, which carried the expanded polyQ (78 glutamine repeats, Q_{78}), were designated as disease flies; flies that expressed the Q_{27} protein were controls and considered normal flies. The untreated flies expressing Q_{78} displayed the shortest survival, and the disease became manifest during the middle of their life span. Cystamine (Cyst), an orally active TGM2 inhibitor, served as positive treatment modality, as it has been effective in mouse model of polyQ disease (Dedeoglu et al., 2002; Karpuj et al., 2002). Cyst had a beneficial effect in this model, although it was not as effective as ZM39923, Me-3,4-dephostatin (Me), or NSC95397 in preventing early death (Figure 4). Vitamin K_3 did not function as an active agent in rescuing the disease flies.

DISCUSSION

TGM2 is being considered as a therapeutic target for the treatment of several diseases. To date, there are few reports of using an orally active TGM2 inhibitor to modify TGM2-based disease process. We screened the LOPAC and Prestwick libraries and discovered candidate compounds that were potent inhibitors of TGM2 protein crosslinking in vitro and inhibited neurodegeneration mediated by a polyQ protein expressed in the CNS. The JAK kinases inhibitors (ZM39923 and ZM449829), EGFR tyrosine kinase inhibitor (tyrphostin 47), phosphatase inhibitors (Me-3,4-dephostatin and NSC95397), and naphthoquinones

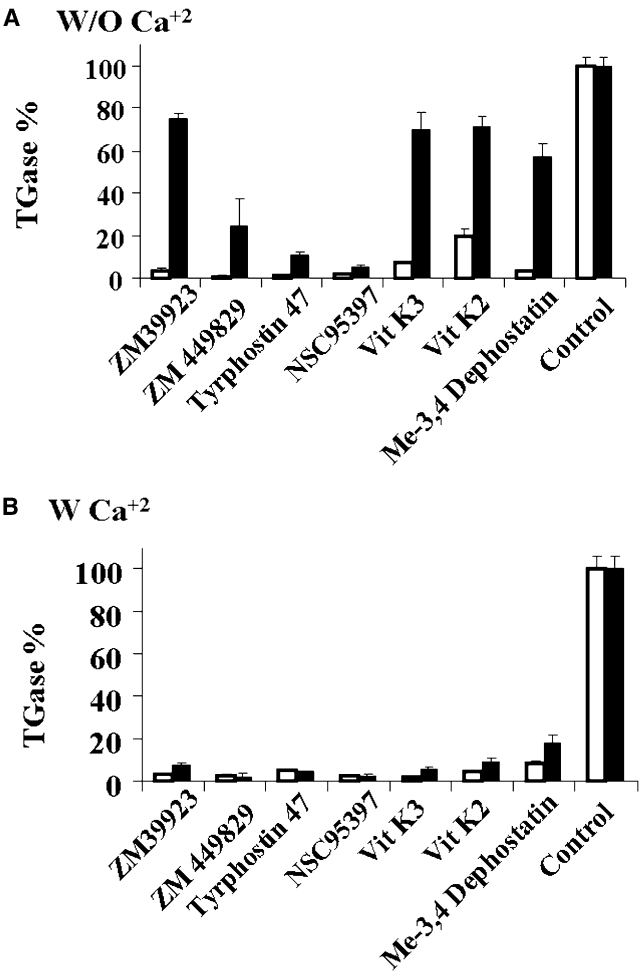


Figure 2. Ca^{+2}-Dependent Inhibition of TGase/TGM2 by Chemical Inhibitors

TGM2 was incubated in the absence (A) or presence (B) of 1 mM Ca^{+2} with inhibitors at concentration that inhibited >95% of TGase activity. The mixture was dialyzed in 41 of 50 mM HEPES (pH 7.5). The incubation mixtures were used to determine TGase activity using BP incorporation assay. The standard deviations were derived from triplicate experiments. Open bar: before dialysis; closed bar: after dialysis.

Table 3. Effects of DTT on Inhibition of TGase/TGM2

	10 mM DTT	W/O DTT
Menadione (Vit. K_3)	> 50	2.2 ± 0.8
Menaquinone (Vit. K_2 or MK4)	> 50	3 ± 2.8
NSC 95397 (N1786)	> 50	1 ± 0.5
Tyrphostin 47 (T7540)	> 50	3 ± 3
ZM 39923 (Z4626)	3 ± 0.5	0.01 ± 0.01
ZM 449829	20 ± 5	0.005 ± 0.005

1. The activity was determined by BP-incorporation assay and represent the average of two triplicate experiments.
2. Data represent the IC_{50} in μM.

Table 4. Effects of DTT on Inhibition of Factor XIIIa

	W DTT	W/O DTT
NSC 95397 (N1786)	> 50	1 ± 0.5
Vitamin K3	> 50	12.5 ± 2
Tyrphostin 47 (T7540)	> 20	3 ± 1
ZM 39923 (Z4626)	10 ± 2	0.025 ± 0.01
ZM 449829	> 20	0.006 ± 0.005

1. The activity was determined by BP-incorporation assay and represent the average of two triplicate experiments.
2. IC_{50} in μM.

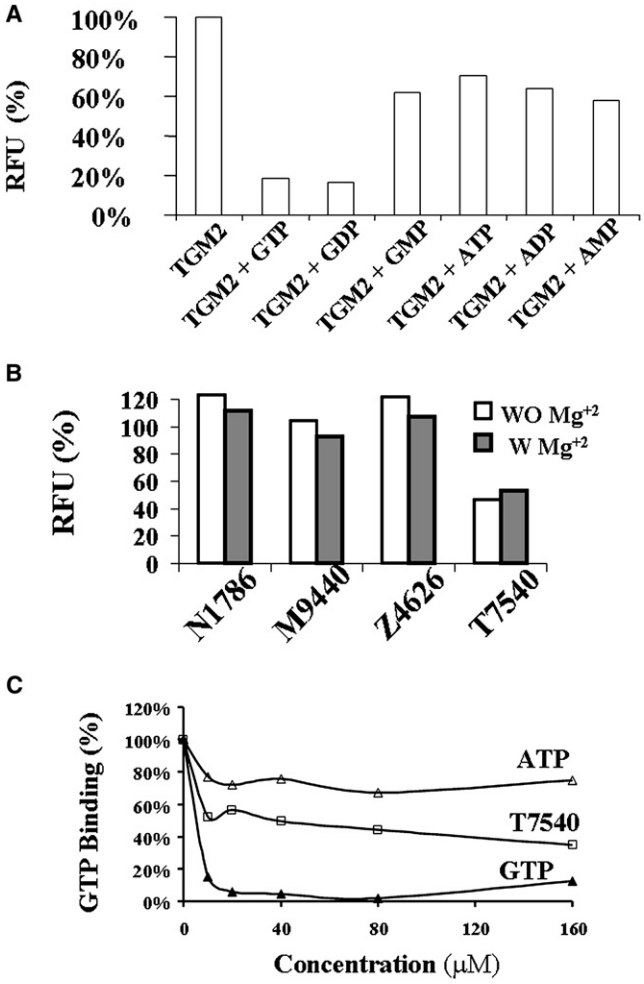


Figure 3. Fluorescent GTP Binding Assay

The fluorescence binding assay was performed in buffer B (50 mM Tris-Cl [pH 7.5], 2 mM DTT, and 1 mM EDTA) containing 400 nM of purified enzyme (TGM2) and 500 nM BODIPY FL-GTP. Buffer B, containing only BODIPY FL-GTP, was used as a reference; the results represented the difference in fluorescence upon binding to the enzymes.

(A) Effects of 150 μM of GTP, GDP, GMP, ATP, ADP, and AMP on BODIPY FL-GTP binding.

(B) Similar to (A) but with inhibitors at 160 μM in the presence (filled bar) or absence (open bar) of 1 mM Mg^{+2}.

(C) Different concentrations of inhibitors (reactive blue 2 and tyrphostin 47), GTP, and ATP were investigated for their effects on BODIPY FL-GTP binding. At each concentration, the binding of inhibitors, GTP, or ATP to BODIPY FL-GTP were used as background value to be subtracted from value obtained with TGM2 binding. There are slight quenching from reactive blue 2 and tyrphostin 47 at concentrations higher than 40 μM.

(vitamin K_3) acted directly on purified TGM2 to inhibit the Ca^{+2}-activated form of TGM2. Our findings suggest that these inhibitors target activated TGM2 and the free thiol groups that are required for an active enzyme.

The specificity of the selected inhibitors was validated through two completely different formats of screening assays—one that used a soluble-phase fluorescent substrate and the other that used a soluble and bound TGM2 substrate. Triton X-100 eliminated the effects of nonspecific small-molecule aggregates (McGovern et al., 2003). The IC_{50} of ZM39923, ZM449829, Me-3,4-dephostatin, tyrphostin 47, NSC95397, and vitamin K_3 were found to have little inhibition and some enhancement after Triton X-100 treatment (Tables 1 and 2). In contrast, GW5074, Rottlerin, β-lapachone, and reactive blue 2 were significantly reduced after Triton X-100 treatment, suggesting that they formed aggregates and were not further characterized in detail. Only the Triton X-100 resistant chemicals were analyzed and found to mediate their inhibition by formation of disulfide bonds.

ZM39923 was found to be a potent inhibitor of TGM2 with IC_{50} of 10 nM and its metabolite; ZM449829 had the lowest IC_{50} value (5 nM). ZM39923 belongs to the class of 2-aminoethyl ketones (or naphthyl ketones) that binds to the ATP-binding site of a tyrosine kinase, Janus kinase 3 (Luo and Laaja, 2004). ZM39923 and ZM449829 target JAK1, JAK3, and epidermal growth factor receptor (EGFR) with reported IC_{50}s in the range of 4.4–7 μM (Luo and Laaja, 2004), whereas TGM2 was inhibited in the nanomolar range. The inhibition by ZM39923 and ZM449829 was not due to aggregate formation or potential chelation of calcium ions, as Ca^{+2} was present at 10,000-fold higher concentrations than ZM39923.

Pinkas et al. (2007) recently crystallized the inhibitor-bound TGM2 and found that a disulfide bond between Cys^{370} and Cys^{371} was involved in keeping the molecule in open conformation. A mechanism that breaks this bond is necessary for formation of the active enzyme. We investigated whether disruption of disulfide bonds by reduction had any effect on the inhibition of TGM2. The potency of inhibitors listed in Table 3 was significantly reduced when DTT was included in the transglutaminase reaction, suggesting that inhibitors promoted formation of disulfide bonds that inactivated the protein. The binding of inhibitors (Table 3) to Ca^{+2}-activated TGM2 could promote disulfide bond formation and induce a conformational change that adopts an open conformation (Pinkas et al., 2007). There is a disulfide bond formation between Cys^{370} and Cys^{371} on inactive inhibitor-bound TGM2 (Pinkas et al., 2007), which locks the protein into an inactive conformation. DTT reversed the inhibition by inhibitors listed in Table 3, suggesting that cysteine thiol(s) in TGM2 might be the target for these inhibitors. Further studies are needed to identify which Cys residue(s) were involved and whether these chemicals lead to specific disulfide bonding that alter the enzyme's function. The inhibition by ZM39923 and ZM449829 was not completely reversed by DTT, suggesting that they are tight binding inhibitors and may have a more complex mechanism of inhibition (Table 3). The inability of these inhibitors to inhibit TGM2 under reducing conditions suggests that they are likely to be less inhibitory to intracellular TGM2 because of the presence of intracellular reducing environment. However, there are many gaps in our knowledge regarding where and when TGM2 is active.

TGM2 has been recognized as a calcium-dependent enzyme for many years (Lorand and Graham, 2003). Dialysis experiments indicated that the chemicals targeted Ca^{+2}-TGM2 (Figure 2). In the absence of Ca^{+2}, there was only 20%–40% inhibition by ZM39923, vitamin K_3, vitamin K_2, and Me-3,4-dephostatin, whereas >90% inhibition was observed by ZM449829, tyrphostin 47, and NSC95397 (Figure 2A). When TGM2 was incubated with chemicals in the presence of Ca^{+2}, all inhibitors were

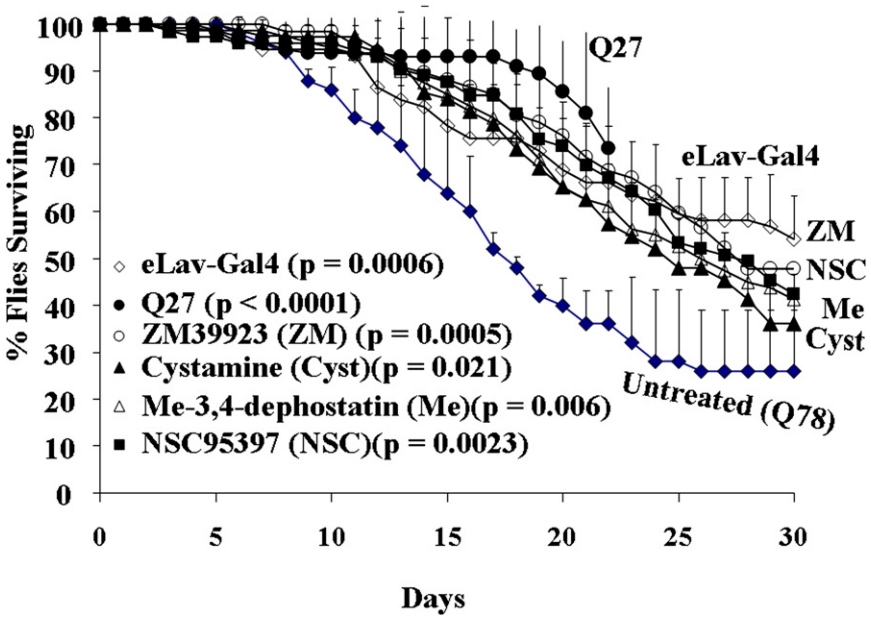


Figure 4. Effects of Chemical Inhibitors in Rescuing *Drosophila* in MJD Model of Neurodegeneration

Chemicals were investigated in these flies at 10 μM. Chemical stocks were diluted to final DMSO concentrations of ≤0.1%. Fly food was replenished every 2–3 days. The number of flies left in the tubes was counted every other day. Untreated flies (Q_{78}) and flies that carried normal length of glutamine repeats (Q_{27}) were used as controls. Each chemical was tested in at least three different tubes, and the survival rate of flies was calculated by dividing the number of flies that survived by the number of starting flies. The log rank p values compare treated groups and the control (untreated) group, as described in Methods. The standard deviations were derived from triplicate experiments. ZM: ZM39923; Cyst: cystamine; Me 3–4: Me-3,4 dephostatin; Q27: elav-Gal4/Q27; Q78: elav-Gal4-Q78.

irreversible (Figure 2B). These data suggest that the inhibitors prefer Ca^{+2}-activated TGM2 as the target and not inactive TGM2. Ca^{+2} induces a conformation that exposes the TGM2 active site (Lorand and Graham, 2003). It is likely that a critical cysteine thiol, either in the active site Cys277 or in the Cys^{370}–Cys^{371} disulfide bond, was made accessible by these inhibitors in Ca^{+2}-TGM2. The reversal of inhibition by DTT suggests that these chemicals interact directly or indirectly with SH groups that modulate catalysis. These chemicals do not alter all forms of TGM2, which may make them potentially less toxic and more selective by inhibiting calcium-active TGM2.

Competitive fluorescent BODIPY FL-GTP binding assay was used to investigate whether kinase inhibitors bind to the same site as GTP (Figure 3). Tyrphostin 47 competed for GTP binding (Figures 3B and 3C) and partially protected TGM2 from proteolysis (Supplemental Data). ZM39923, NSC95397, and Me-3,4-dephostatin did not compete directly for GTP binding and may bind to another site that plays a role in regulating catalysis.

Two chemicals designed to target either tyrosine kinases (tyrphostin 47) or tyrosine phosphatases (NSC95397 and Me-3,4-dephostatin) were also found to inhibit TGM2 but did so less potently than ZM39923, which was designed to target Janus kinase 3, suggesting that TGM2 shares a similar kinase-like binding site (Faaland et al., 1991; Liu, 2003; Peyregne et al., 2005; Prevost et al., 2003; Suzuki et al., 2001; Tamura et al., 2000; Wolbring et al., 1994). Tyrphostin compounds target tyrosine kinases by binding to the kinase substrate binding site (Nowak et al., 1997). The tyrosine kinase inhibitors used to inhibit TGM2 are used at ~100 μM in most cellular studies (Bain et al., 2003; Davies et al., 2000); however, our findings suggest Ca-TGM2 is a target at lower concentrations. The fact that kinase inhibitors bind and inhibit TGM2 is not unexpected because TGM2 can bind ATP and function as a kinase (Mishra and Murphy, 2004a, 2006). These findings also indicate that tyrosine itself can be used as a scaffold in the design of TGase/TGM2 inhibitors. It was reported that synthetic tyrosine melanin was a good inhibitor of TGase/TGM2 (Ikura et al., 2002).

Several chemicals identified as TGM2 inhibitors were designed to inhibit the kinases and phosphatases in the EGFR pathway. ZM39923, ZM449829, and NSC95397 inhibit the Cdc25 phosphatase (Ham et al., 1998), which also targets EGFR. EGFR is also the target of tyrphostin 47 (Faaland et al., 1991). Although vitamin K_3 does not bind to tyrosine kinase directly, it is known to activate EGFR tyrosine kinase through inactivation of an unknown regulating tyrosine phosphatase (Klotz et al., 2002). Furthermore, it is known that EGF induces the expression of and activates TGM2 in human breast cancer cell lines (Antonyak et al., 2004). Accordingly, we are currently testing the effects of these inhibitors on TGM2 expression in breast cancer.

Several 1,4-naphthoquinones compounds (menadione, also called vitamin K_3, vitamin K_2 and NSC 95397) were found to be TGM2 inhibitors. Quinones represent the largest class of quinoid compounds, which are widely distributed in nature (Abdelmohsen et al., 2004; Watanabe et al., 2004). Many quinones have multiple biological functions, including antitumor and carcinogenic activities. Vitamin K_3 (2-methyl-1,4-naphthoquinone) exhibits a broad spectrum of anticancer activity (Lamson and Plaza, 2003). The vitamin K family of molecules comprises the natural forms, vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinones), as well as the synthetic form, vitamin K_3.

The 1,4-naphthoquinone scaffold present in NSC 95397 and vitamin K_3 was also found to be an irreversible cysteine protease inhibitor (Valente et al., 2007). The enzymatic mechanism of TGM2 is similar to that employed by cysteine proteases, involving a catalytic triad of Cys, His, and Asp (Liu et al., 2002; Pedersen et al., 1994). The cysteine thiol group reacts with glutamyl-containing substrates to form a reactive thioester intermediate, from which the acyl group is transferred to an amine substrate (Lorand and Graham, 2003). These data suggest that these inhibitors may act directly on the catalytic triad of TGM2.

In contrast, *p*-benzoquinone (BQ), a strong alkylating quinone, and an exclusive redox-cycler 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) (Abdelmohsen et al., 2003) did not inhibit TGM2 activity, suggesting a specific interaction of vitamin K_3, vitamin K_2, NSC 95397, and ZM39923 that was not based solely on nonspecific alkylation. The target of NSC95397, Cdc25A, a dual-specificity protein phosphatase, plays a critical role in cell cycle progression (Wang et al., 2002). Cdc25A interacts with EGFR both physically and functionally in Hep3B human hepatoma cells (Wang et al., 2002).

The inhibitors identified in this study inhibited the plasma transglutaminase FXIIIa with IC_{50} values comparable to TGM2. In addition, reducing disulfide bond formations prevented the chemicals from functioning as inhibitors. This suggests they function by a similar mechanism. FXIII A chain (FXIIIA) is a zymogen and shares 39% amino acid identity with human TGM2 with the highest degree in the TGase active site pocket and calcium binding site (Lorand and Graham, 2003). Despite their similarity, FXIIIa has not been reported to bind nucleotides (Lorand and Graham, 2003). However, it has been reported that FXIIIa has kinase activity (Mishra, 2004b), suggesting that it contains a nucleotide binding site. Alternatively, there may be sufficient homology in FXIIIa to promote the binding that leads to the inhibition at the active site or other SH-dependent site.

The capacity of the TGM2 inhibitors to display in vivo activity was tested in a *Drosophila* model of expanded polyQ disease (Figure 4). Most of the inhibitors tested showed statistically significant and beneficial effects in this model, with ZM39923 performing best in rescuing Q_{78} flies from early death. However, vitamin K_3, a TGM2 inhibitor, did not show any statistically significant beneficial effects in this model. This may relate to its inefficiency at entering the CNS, its ability to inhibit species-specific *Drosophila* TGM2, or another mechanism. The underlying mechanism of action of each inhibitor will require further investigation. Because these compounds were initially designed to target other kinases, inhibition of TGM2 activity may not solely explain the beneficial effects they have in vivo. One possible in vivo mechanism of action to consider is that when the kinase domain of TGM2 was occupied by these inhibitors, disulfide bonds formed that kept TGM2 in a conformation that inhibited the crosslinking of Q_{78}-containing protein. Current research underway will further define how TGM2 inhibitors discovered in this study function to alter TGM2 catalysis.

SIGNIFICANCE

This study documents that there are inhibitors of TGM2 in drug libraries. These inhibitors target the Ca^{+2}-activated form of TGM2. Inhibition depends on disulfide bond formation because reducing agents interfere with the inhibition. Oral administration of these agents prevented the premature death of *Drosophila* expressing polyQ proteins. To our knowledge, this is the first example of screening existing drug libraries for TGM2 inhibitors and may lead to further advances in development of orally active TGM2 inhibitors. As some older drugs have been found to have new uses (Chong and Sullivan, 2007), our data are significant in providing important leads to develop more specific inhibitors to TGM2 that can be applied to the devastating neurodegenerative disease mediated by polyQ-expressing proteins. Our findings may also aid in the development of drugs inhibiting TGM2-dependent pathology associated with tissue fibrosis, aging, and organ failure.

EXPERIMENTAL PROCEDURES

Materials

Monoclonal antibodies to human TGM2 (Cub7402) were purchased from Lab Vision (Fremont, CA). A mouse monoclonal antibody to TGM2, Cub7401, was obtained as a gift from Dr. P.J. Birckbichler (Birckbichler et al., 1985). 5-Biotinamido pentylamine (BP) was obtained from Pierce (Rockford, IL). The LOPAC and Prestwick libraries were provided by the Duke Center for Chemical Biology. Chemicals chosen for further characterization were purchased from Sigma-Aldrich or Tocris (Ellisville, MO). Chemicals were dissolved in either 100% DMSO or 95% ethanol (vitamin K_3 and vitamin K_2) and stored at −20°C.

Expression and Purification of Recombinant TGM2 and Blood Coagulation Factor XIII A Chains (FXIIIA)

The recombinant TGM2 and FXIIIA were expressed and purified in *E. coli* as a GST fusion protein as described previously (Lai et al., 1994, 1998). Protein concentrations were quantified using the Bradford method (Bio-Rad; Hercules, CA). The recombinant proteins were purified in the presence of reducing agent DTT, dialyzed against 50 mM Tris-acetate buffer (pH 7.5), 1 mM DTT, and 15% glycerol, and stored at −80°C until ready for use.

Transglutaminase BP Incorporation Assay

TGase/TGM2 activity was determined by quantifying the incorporation of BP into *N,N′*-dimethylcasein in a microtiter plate as described previously (Slaughter et al., 1992). Zymogen FXIIIA was activated with 10 U/ml of thrombin to become FXIIIa at 37°C for 20 min as described (Lai et al., 1994). The amount of BP incorporated into the casein was determined after 45 min incubation at 37°C and color developed using streptavidin-conjugated alkaline phosphatase and para-nitrophenyl phosphate (pNPP) (Slaughter et al., 1992). For IC_{50} determination, all assays were determined in the presence of 1 mM BP. For accurate measurement of IC_{50}, narrower range concentration of inhibitors was chosen for potent inhibitors. To prevent oxidation, all chemicals were freshly diluted from −20°C stock solution before each assay.

Transglutaminase Fluorescence Assay

The TGase fluorescence assay was used to quantify the incorporation of BOC-K-EDA-Dansyl (KXD, a K substrate) into *N,N′*-dimethylcasein (NMC, a Q substrate) (Case and Stein, 2007). In the absence of potential inhibitors, the assay had a 3-fold increase in fluorescence intensity (λ_{Ex} 340 nm; λ_{Em} 520 nm) when BOC-K-EDA-Dansyl was crosslinked to *N,N′*-dimethylcasein.

Fluorescent GTP Binding Assay

The binding assay was performed using a fluorescent GTP analog, BODIPY FL-GTP (Molecular Probes and Invitrogen; Carlsbad, CA) essentially as described (Datta et al., 2006; McEwen et al., 2002). All fluorescence measurements were performed in a cuvette and determined in Molecular Device's (Sunnyvale, CA) SpectraMax M2e fluorescence reader. The experiments were carried out in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM DTT and 1 mM EDTA. The detection for BODIPY FL-GTP was set at λ_{Ex} 485 nm and λ_{Em} 520 nm with a λ_{495nm} cutoff.

Trypsin Proteolysis of Recombinant TGM2

Purified recombinant GST-TGM2 (1 μg) was incubated with 0.1 μg of trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated and high-pressure liquid chromatography purified (Calbiochem Biosciences; San Diego, CA) in the presence of 100 μM free GTP or chemicals, and incubated at 37°C for 1 hr (Lai et al., 1998). The reaction was stopped by the addition of SDS-PAGE loading buffer. Samples were separated by SDS-PAGE and analyzed by immunoblotting using the monoclonal antibody Cub7402 against human TGM2.

Effects of Inhibitors in a *Drosophila melanogaster* Model of Machado-Joseph Disease

The transgenic fly line bearing the UAS-MJDtr-Q_{78} transgene has been described previously (Warrick et al., 1998). These flies express a carboxy-terminal fragment of the MJD protein containing 78 glutamines with a hemaglutinin epitope tag at the N terminus, under the control of the GAL4-UAS system. Fly culture and crosses were performed under standard conditions at 25°C. The fly stocks, including the fly line bearing the *elav*-GAL4 transgene, were obtained from Model System of Genomics (Department of Biology, Duke University). Instant *Drosophila* medium (Carolina Biological Supply; Burlington, NC) was used. We used flies expressing expanded polyglutamine (78 glutamine repeats, designated as Q_{78}) as disease flies and flies carrying Q_{27} domain as normal flies (Popiel et al., 2007). Compounds were investigated in these flies at 10 μM. Chemical stocks were diluted to 10 mM in DMSO and 1:1,000 with sterile 5% sucrose solution. Then 3.33 parts solution was added to one part solid flake food (formula-4-24; Carolina Biological Supply) and mixed. We found 0.7% DMSO is toxic at some level for flies, so the final DMSO concentrations were $\leq$0.1%. Fly food was replenished every 2–3 days. The number of flies left in the tubes was counted every other day. Untreated flies (Q_{78}) and living flies that carried normal length of glutamine repeats (Q_{27}) were used as controls. Each chemical was tested for at least 50 flies divided in three or more separate test tubes (i.e., triplicate for each chemical). The survival rate of flies was calculated as a percentage of flies remaining in the tubes at the end of test period. Survival functions were modeled using the Kaplan-Meier method as implemented in SAS version 9.1.3 (SAS; Cary, NC). Comparisons between treatment groups were made using the log rank test. Statistical significance was declared at $p < 0.05$.

SUPPLEMENTAL DATA

Supplemental Data include one figure and five tables and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/9/969/DC1/.

ACKNOWLEDGMENTS

The technical assistance of W. Li, Ashton Lai, and Alex Tremont is greatly appreciated. We thank the Duke Center for Biology for providing the LOPAC and Preswick libraries for initial screening. This research was funded in part by National Institutes of Health Grants HL072184 (to C.S.G.) and NS050541 (to T.S.L.).

Received: November 13, 2007
Revised: July 16, 2008
Accepted: July 23, 2008
Published: September 19, 2008

REFERENCES

Abdelmohsen, K., Gerber, P.A., von Montfort, C., Sies, H., and Klotz, L.O. (2003). Epidermal growth factor receptor is a common mediator of quinone-induced signaling leading to phosphorylation of connexin-43: role of glutathione and tyrosine phosphatases. J. Biol. Chem. *278*, 38360–38367.

Abdelmohsen, K., Patak, P., Von Montfort, C., Melchheier, I., Sies, H., and Klotz, L.O. (2004). Signaling effects of menadione: from tyrosine phosphatase inactivation to connexin phosphorylation. Methods Enzymol. *378*, 258–272.

Achyuthan, K.E., and Greenberg, C.S. (1987). Identification of a guanosine triphosphate-binding site on guinea pig liver transglutaminase. Role of GTP and calcium ions in modulating activity. J. Biol. Chem. *262*, 1901–1906.

Andringa, G., Lam, K.Y., Chegary, M., Wang, X., Chase, T.N., and Bennett, M.C. (2004). Tissue transglutaminase catalyzes the formation of alpha-synuclein crosslinks in Parkinson's disease. FASEB J. *18*, 932–934.

Antonyak, M.A., Miller, A.M., Jansen, J.M., Boehm, J.E., Balkman, C.E., Wakshlag, J.J., Page, R.L., and Cerione, R.A. (2004). Augmentation of tissue transglutaminase expression and activation by epidermal growth factor inhibit doxorubicin-induced apoptosis in human breast cancer cells. J. Biol. Chem. *279*, 41461–41467.

Arrasate, M., Mitra, S., Schweitzer, E.S., Segal, M.R., and Finkbeiner, S. (2004). Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature *431*, 805–810.

Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003). The specificities of protein kinase inhibitors: an update. Biochem. J. *371*, 199–204.

Birckbichler, P.J., Upchurch, H.F., Patterson, M.K., Jr., and Conway, E. (1985). A monoclonal antibody to cellular transglutaminase. Hybridoma *4*, 179–186.

Case, A., and Stein, R.L. (2003). Kinetic analysis of the action of tissue transglutaminase on peptide and protein substrates. Biochemistry *42*, 9466–9481.

Case, A., and Stein, R.L. (2007). Kinetic analysis of the interaction of tissue transglutaminase with a nonpeptidic slow-binding inhibitor. Biochemistry *46*, 1106–1115.

Chong, C.R., and Sullivan, D.J., Jr. (2007). New uses for old drugs. Nature *448*, 645–646.

Datta, S., Antonyak, M.A., and Cerione, R.A. (2006). Importance of Ca(2+)-dependent transamidation activity in the protection afforded by tissue transglutaminase against doxorubicin-induced apoptosis. Biochemistry *45*, 13163–13174.

Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. *351*, 95–105.

Dedeoglu, A., Kubilus, J.K., Jeitner, T.M., Matson, S.A., Bogdanov, M., Kowall, N.W., Matson, W.R., Cooper, A.J., Ratan, R.R., Beal, M.F., et al. (2002). Therapeutic effects of cystamine in a murine model of Huntington's disease. J. Neurosci. *22*, 8942–8950.

Faaland, C.A., Mermelstein, F.H., Hayashi, J., and Laskin, J.D. (1991). Rapid uptake of tyrphostin into A431 human epidermoid cells is followed by delayed inhibition of epidermal growth factor (EGF)-stimulated EGF receptor tyrosine kinase activity. Mol. Cell. Biol. *11*, 2697–2703.

Folk, J.E. (1983). Mechanism and basis for specificity of transglutaminase-catalyzed epsilon-(gamma-glutamyl) lysine bond formation. Adv. Enzymol. Relat. Areas Mol. Biol. *54*, 1–56.

Greenberg, C.S., Birckbichler, P.J., and Rice, R.H. (1991). Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. FASEB J. *5*, 3071–3077.

Ham, S.W., Park, J., Lee, S.J., Kim, W., Kang, K., and Choi, K.H. (1998). Naphthoquinone analogs as inactivators of cdc25 phosphatase. Bioorg. Med. Chem. Lett. *8*, 2507–2510.

Ikura, K., Otomo, C., Natsuka, S., Ichikawa, A., Wakamatsu, K., Ito, S., and Taoguchi, S. (2002). Inhibition of transglutaminase by synthetic tyrosine melanin. Biosci. Biotechnol. Biochem. *66*, 1412–1414.

Karpuj, M.V., Becher, M.W., Springer, J.E., Chabas, D., Youssef, S., Pedotti, R., Mitchell, D., and Steinman, L. (2002). Prolonged survival and decreased abnormal movements in transgenic model of Huntington disease, with administration of the transglutaminase inhibitor cystamine. Nat. Med. *8*, 143–149.

Klotz, L.O., Patak, P., Ale-Agha, N., Buchczyk, D.P., Abdelmohsen, K., Gerber, P.A., von Montfort, C., and Sies, H. (2002). 2-Methyl-1,4-naphthoquinone, vitamin K(3), decreases gap-junctional intercellular communication via activation of the epidermal growth factor receptor/extracellular signal-regulated kinase cascade. Cancer Res. *62*, 4922–4928.

Konno, T., Morii, T., Hirata, A., Sato, S., Oiki, S., and Ikura, K. (2005). Covalent blocking of fibril formation and aggregation of intracellular amyloidgenic proteins by transglutaminase-catalyzed intramolecular cross-linking. Biochemistry *44*, 2072–2079.

Lai, T.S., Achyuthan, K.E., Santiago, M.A., and Greenberg, G.S. (1994). Carboxyl-terminal truncation of recombinant factor XIII A-chains. Characterization of minimum structural requirement for transglutaminase activity. J. Biol. Chem. *269*, 24596–24601.

Lai, T.S., Slaughter, T.F., Peoples, K.A., Hettasch, J.M., and Greenberg, C.S. (1998). Regulation of human tissue transglutaminase function by magnesium-nucleotide complexes. Identification of distinct binding sites for Mg-GTP and Mg-ATP. J. Biol. Chem. *273*, 1776–1781.

Lai, T.S., Tucker, T., Burke, J.R., Strittmatter, W.J., and Greenberg, C.S. (2004). Effect of tissue transglutaminase on the solubility of proteins containing expanded polyglutamine repeats. J. Neurochem. *88*, 1253–1260.

Lamson, D.W., and Plaza, S.M. (2003). The anticancer effects of vitamin K. Altern. Med. Rev. *8*, 303–318.

Liu, G. (2003). Protein tyrosine phosphatase 1B inhibition: opportunities and challenges. Curr. Med. Chem. *10*, 1407–1421.

Liu, S., Cerione, R.A., and Clardy, J. (2002). Structural basis for the guanine nucleotide-binding activity of tissue transglutaminase and its regulation of transamidation activity. Proc. Natl. Acad. Sci. U.S.A. *99*, 2743–2747.

Lorand, L., and Graham, R.M. (2003). Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat. Rev. Mol. Cell Biol. *4*, 140–156.

Luo, C., and Laaja, P. (2004). Inhibitors of JAKs/STATs and the kinases: a possible new cluster of drugs. Drug Discov. Today *9*, 268–275.

Malorni, W., Farrace, M.G., Rodolfo, C., and Piacentini, M. (2008). Type 2 transglutaminase in neurodegenerative diseases: the mitochondrial connection. Curr. Pharm. Des. *14*, 278–288.

Mastroberardino, P.G., Iannicola, C., Nardacci, R., Bernassola, F., De Laurenzi, V., Melino, G., Moreno, S., Pavone, F., Oliverio, S., Fesus, L., and Piacentini, M. (2002). 'Tissue' transglutaminase ablation reduces neuronal death and prolongs survival in a mouse model of Huntington's disease. Cell Death Differ. *9*, 873–880.

McEwen, D.P., Gee, K.R., Kang, H.C., and Neubig, R.R. (2002). Fluorescence approaches to study G protein mechanisms. Methods Enzymol. *344*, 403–420.

McGovern, S.L., Helfand, B.T., Feng, B., and Shoichet, B.K. (2003). A specific mechanism of nonspecific inhibition. J. Med. Chem. *46*, 4265–4272.

Mishra, S., and Murphy, L.J. (2004a). Tissue transglutaminase has intrinsic kinase activity: identification of transglutaminase 2 as an insulin-like growth factor-binding protein-3 kinase. J. Biol. Chem. *279*, 23863–23868.

Mishra, S., and Murphy, L.J. (2004b). Transglutaminase has intrinsic kinase activity. United States Patent 7090971.

Mishra, S., and Murphy, L.J. (2006). The p53 oncoprotein is a substrate for tissue transglutaminase kinase activity. Biochem. Biophys. Res. Commun. *339*, 726–730.

Muma, N.A. (2007). Transglutaminase is linked to neurodegenerative diseases. J. Neuropathol. Exp. Neurol. *66*, 258–263.

Murthy, S.N., Lomasney, J.W., Mak, E.C., and Lorand, L. (1999). Interactions of G(h)/transglutaminase with phospholipase Cdelta1 and with GTP. Proc. Natl. Acad. Sci. U.S.A. *96*, 11815–11819.

Nagai, Y., Inui, T., Popiel, H.A., Fujikake, N., Hasegawa, K., Urade, Y., Goto, Y., Naiki, H., and Toda, T. (2007). A toxic monomeric conformer of the polyglutamine protein. Nat. Struct. Mol. Biol. *14*, 332–340.

Nakaoka, H., Perez, D.M., Baek, K.J., Das, T., Husain, A., Misono, K., Im, M.J., and Graham, R.M. (1994). Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function. Science *264*, 1593–1596.

Nowak, F., Jacquemin-Sablon, A., and Pierre, J. (1997). Epidermal growth factor receptor signaling cascade as target for tyrphostin (RG 50864) in epithelial cells. Paradoxical effects on mitogen-activated protein kinase kinase and mitogen-activated protein kinase activities. Biochem. Pharmacol. *53*, 287–298.

Pedersen, L.C., Yee, V.C., Bishop, P.D., Le Trong, I., Teller, D.C., and Stenkamp, R.E. (1994). Transglutaminase factor XIII uses proteinase-like catalytic triad to crosslink macromolecules. Protein Sci. *3*, 1131–1135.

Peyregne, V.P., Kar, S., Ham, S.W., Wang, M., Wang, Z., and Carr, B.I. (2005). Novel hydroxyl naphthoquinones with potent Cdc25 antagonizing and growth inhibitory properties. Mol. Cancer Ther. *4*, 595–602.

Pinkas, D.M., Strop, P., Brunger, A.T., and Khosla, C. (2007). Transglutaminase 2 undergoes a large conformational change upon activation. PLoS Biol. *5*, e327.

Popiel, H.A., Nagai, Y., Fujikake, N., and Toda, T. (2007). Protein transduction domain-mediated delivery of QBP1 suppresses polyglutamine-induced neurodegeneration in vivo. Mol. Ther. *15*, 303–309.

Prevost, G.P., Brezak, M.C., Goubin, F., Mondesert, O., Galcera, M.O., Quaranta, M., Alby, F., Lavergne, O., and Ducommun, B. (2003). Inhibitors of the CDC25 phosphatases. Prog. Cell Cycle Res. *5*, 225–234.

Slaughter, T.F., Achyuthan, K.E., Lai, T.S., and Greenberg, C.S. (1992). A microtiter plate transglutaminase assay utilizing 5-(biotinamido)pentylamine as substrate. Anal. Biochem. *205*, 166–171.

Suzuki, T., Hiroki, A., Watanabe, T., Yamashita, T., Takei, I., and Umezawa, K. (2001). Potentiation of insulin-related signal transduction by a novel protein-tyrosine phosphatase inhibitor, Et-3,4-dephostatin, on cultured 3T3–L1 adipocytes. J. Biol. Chem. *276*, 27511–27518.

Tamura, K., Southwick, E.C., Kerns, J., Rosi, K., Carr, B.I., Wilcox, C., and Lazo, J.S. (2000). Cdc25 inhibition and cell cycle arrest by a synthetic thioalkyl vitamin K analogue. Cancer Res. *60*, 1317–1325.

Tucholski, J., Kuret, J., and Johnson, G.V. (1999). Tau is modified by tissue transglutaminase in situ: possible functional and metabolic effects of polyamination. J. Neurochem. *73*, 1871–1880.

Valente, C., Moreira, R., Guedes, R.C., Iley, J., Jaffar, M., and Douglas, K.T. (2007). The 1,4-naphthoquinone scaffold in the design of cysteine protease inhibitors. Bioorg. Med. Chem. *15*, 5340–5350.

Wang, Z., Wang, M., Lazo, J.S., and Carr, B.I. (2002). Identification of epidermal growth factor receptor as a target of Cdc25A protein phosphatase. J. Biol. Chem. *277*, 19470–19475.

Warrick, J.M., Paulson, H.L., Gray-Board, G.L., Bui, Q.T., Fischbeck, K.H., Pittman, R.N., and Bonini, N.M. (1998). Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in Drosophila. Cell *93*, 939–949.

Watanabe, N., Dickinson, D.A., Liu, R.M., and Forman, H.J. (2004). Quinones and glutathione metabolism. Methods Enzymol. *378*, 319–340.

Wolbring, G., Hollenberg, M.D., and Schnetkamp, P.P. (1994). Inhibition of GTP-utilizing enzymes by tyrphostins. J. Biol. Chem. *269*, 22470–22472.

