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Chemistry and Biology of Dihydroisoxazole Derivatives: Selective Inhibitors of Human Transglutaminase 2

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Summary

3-Halo-4,5-dihydroisoxazoles are attractive warheads for the selective inhibition of nucleophilic active sites in biological systems. A series of 3-bromo-4,5-dihydroisoxazole compounds were prepared and tested for their ability to irreversibly inhibit human transglutaminase 2 (TG2), an enzyme that plays an important role in the pathogenesis of diverse disorders including Celiac Sprue and certain types of cancers. Several compounds showed high specificity for human TG2 ($k_{inh}/K_I > 2000$ min^{-1}M^{-1}) but essentially no reactivity ($k < 1$ min^{-1}M^{-1}) toward physiological thiols such as glutathione. The pharmacokinetic and pharmacodynamic properties of a prototype dihydroisoxazole inhibitor, 1b, were evaluated; in mice the compound showed good oral bioavailability, short serum half-life and efficient TG2 inhibition in small intestinal tissue, and low toxicity. It also showed excellent synergism with *N,N′*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU, carmustine) against refractory glioblastoma tumors in mice. A fluorescent dihydroisoxazole inhibitor 5 facilitated microscopic visualization of TG2 endocytosis from the extracellular surface of HCT-116 cells. Together, these findings demonstrate the promise of dihydroisoxazole compounds as probes for the biology of TG2 and its role in human disease.

Introduction

Tissue transglutaminase (also called transglutaminase 2 [TG2]) is a calcium-dependent enzyme that catalyzes crosslinking between selected glutamine γ-carboxamide groups and lysine ε-amino groups in proteins [1–3]. TG2 is ubiquitously expressed in different types of tissues and is reported to have extracellular as well as intracellular functions. Outside the cell TG2 anchors onto the extracellular matrix, crosslinks fibronectin and related proteins, and is thereby involved in cell movement, adhesion, and proliferation. Intracellular TG2 loses enzyme activity when bound to GTP, but functions as a G protein in the phospholipase C signal transduction cascade. TG2 has considerable pharmacological significance, as it is believed to play a crucial role in the pathogenesis of diverse human disorders [4], including Celiac Sprue, neurological disorders such as Huntington's, Alzheimer's, and Parkinson's diseases, and certain types of cancers. The medicinal attractiveness of this protein target is underscored by the observation that TG2 knockout mice are normal, lacking developmental, physiological, or reproductive defects. Indeed, when TG2 knockout mice are crossed with Huntington's disease-prone R6/1 mice, the resulting hybrids exhibit reduced neuronal cell death and prolonged survival [5]. Thus, given the fundamental and practical interest in TG2 biology, the development of small molecule inhibitors capable of modulating its function in cell-based assays, animals, and eventually humans would be desirable. Here we describe the synthesis, in vitro activity, and in vivo evaluation of a class of inhibitors with considerable potential for such studies.

The catalytic mechanism of TG2 is similar to that employed by cysteine proteases, involving a catalytic triad of cysteine, histidine, and aspartate. The cysteine thiol group reacts with a glutamyl substrate to form a reactive thioester intermediate, from which the acyl group is transferred to an amine substrate (Figure 1). Several electrophilic functional groups have been used as warheads to inactivate cysteine-dependent enzymes by covalent modification of an active thiol group [6–11]. One heterocyclic compound used as an irreversible inhibitor of transglutaminases is 3-halo-4,5-dihydroisoxazole, which is the reactive moiety of acivicin, a naturally occurring glutamine analog with low in vivo toxicity. Previous work has shown that decarboxylated acivicin derivatives (**1**) are potent inhibitors of bovine epidermal transglutaminase [11]. As part of our ongoing efforts to develop dihydroisoxazole-based TG2 inhibitors [12], we have synthesized analogs of compound **1**. Here we report their structure-activity relationships against human TG2. We also demonstrate that a lead compound from this series sensitizes tumor cell lines toward clinically relevant proapoptotic agents, is well tolerated in rodents, and shows excellent synergism with *N,N′*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU, carmustine) against refractory glioblastoma tumors in mice.

Results

Structure-Activity Relationships

Synthesis and TG2 Inhibitory Activity of Dihydroisoxazole Inhibitors

Compound **1** and its analogs (Table 1) were prepared by coupling suitably functionalized amino acids using standard carbodiimide chemistry to 3-bromo-5-aminomethyl-4,5-dihydroisoxazole or 3-bromo-5-methylaminomethyl-4,5-dihydroisoxazole (Figure 2), which in turn

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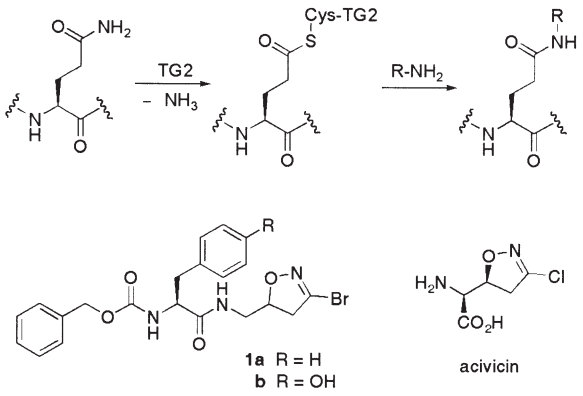


Figure 1. The Enzymatic Action of TG2 and Chemical Structures of the TG2 Inhibitors

See text for details.

were synthesized by [3+2] cycloaddition of bromonitrile oxide with corresponding allyl amines [13]. Using the same cycloaddition reaction, compound **2a** was prepared directly from the corresponding allyl ester.

Prepared dihydroisoxazole compounds were assayed against recombinant human TG2 using an enzyme coupled assay [14, 15], where glutamate dehydrogenase was used to couple ammonium ion, a product of the TG2-catalyzed reaction, to α-ketoglutarate to yield glutamate and the simultaneous oxidation of NADH to NAD^+ was monitored at 340 nm to follow the reaction progress. All the tested compounds exhibited time-dependent inactivation of TG2 and kinetic parameters were obtained by plotting the reaction progress curve against theoretical equations of the irreversible inhibition model (Figure 3) [12].

The assay results showed that compounds **1a** and **1b** are also reasonably active against human TG2, although their specificity (k_{inh}/K_I) is significantly less than the reported values against bovine epidermal transglutaminase [11]. Several analogs were prepared to assess the structure-activity relationships of **1**. Changing the bulky benzyloxycarbonyl (Cbz) group on the α-amino group to a small acetyl group (**3a**) decreased the inhibition activity. Similar results were observed when the secondary amide group connecting the dihydroisoxazole ring to the rest of the molecule was replaced with an ester or a tertiary amide group in compounds **2a** and **2b**, respectively. Based on these results, the Cbz and amide groups were fixed and the side chain of the amino acid moiety was varied using different amino acids (**1c–1i**). The analog derived from tryptophan or γ-substituted aspartic acid retained potent inhibition activity against TG2 while the alanine or D-phenylanine derivatives exhibited significantly reduced activity. The assay results for this series of compounds indicate that the bulky side chain and its stereochemistry are important for the inhibitor activity. In another series of compounds (**3b–3f**) with different aryl carbamate groups, K_I decreased at least 5-fold compared with **1b** and dioxobenzothiophenyl derivative **4** showed the most promising inhibition activity.

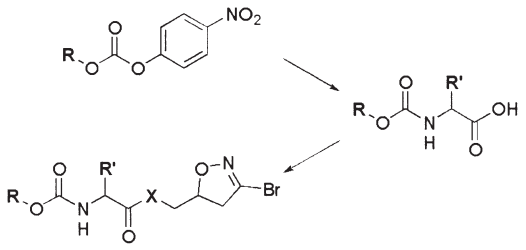


Figure 2. Synthesis of Dihydroisoxazole Derivatives

See text for details.

Reactivity of Dihydroisoxazole Inhibitors toward Alternative Physiological Nucleophiles

The stability of the 3-bromo-4,5-dihydroisoxazole group, the electrophile responsible for the irreversible inhibition, was tested against a representative cysteine thiol group. The reduced form of glutathione (GSH) is probably the most abundant physiological thiol (around 5 mM in animal cells) with a pKa value (8.66). When the solution of **1b** (0.1 mM in a 200 mM MOPS buffer with 3.3% DMSO) was monitored using HPLC for 9 hr of incubation with 5 mM GSH at pH 7.2, no changes were detected in the concentration of **1b** and the same result was observed at pH 9.0. This finding ($k < 1\ min^{-1}M^{-1}$) suggests that the dihydroisoxazole warhead is likely to retain reasonable stability in physiological systems, even at a pH where some amount of the thiol is present in the thiolate form.

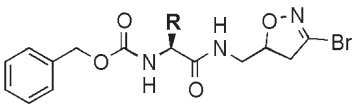
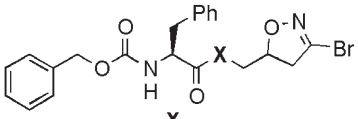
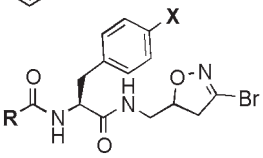
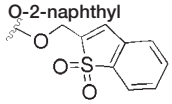
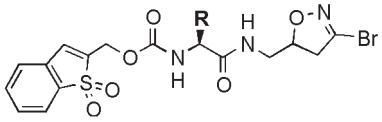
Other dihydroisoxazole compounds tested showed similar stabilities with **1b** except compounds **3f** and **4**, which have a dioxobenzothiophene group. When incubated with 5 mM GSH at pH 7.2, the latter compounds decomposed almost completely within 10 min to multiple products, which have not been characterized fully yet. No decomposition was observed in solutions without the thiol added. An analog of compound **3f**, which has a tyrosine methyl ester group instead of the dihydroisoxazole methyl amide, also decomposed quickly in the presence of GSH confirming that the dioxobenzothiophene group is responsible for the instability. However, this control compound has insignificant TG2 inhibition activity, suggesting that the TG2 inhibitory activities of **3f** and **4** are not correlated with their reactivity toward simple nucleophiles.

Biology of Dihydroisoxazole Inhibitors

Activity of 1b against Tumor Cell Lines in Culture

Studies with competitive TG2 substrate analogs have shown that inhibition of TG2 sensitizes certain tumor cells to death induced by proapoptotic agents ([16], L.Y. et al., unpublished data). To demonstrate chemosensitization activity of the dihydroisoxazole inhibitors described in this report, a cell-based assay was developed using HCT-116, a human colon cancer cell line, and epothilone C, a member of a promising class of tubulin binding anticancer agents (see for example [17–19]). As shown in Figure 4, incubation of HCT-116 with increasing levels of **1b** in the 1–100 μM range reduced the GI_{50} for epothilone C by >2-fold. When used by itself, no cytotoxicity was observed for **1b** at concentra-

Table 1. Dissociation Constants (K_I) and Inhibition Rate Constants (k_{inh}) of Dihydroisoxazole Inhibitors of Human TG2

			K_I (mM)	k_{inh} (min^{-1})	k_{inh}/K_I ($min^{-1}M^{-1}$)
	**1a**	R = $CH_2C_6H_5$	0.74	1.3	1900
	1b	$CH_2C_6H_4$-*p*-OH	0.42	0.86	2000
	1c	$CH_2C_6H_4$-*p*-F	0.43	0.42	980
	1d	$CH_2C_6H_4$-*m*-F	0.39	0.84	2200
	1e	CH_2-3-(5-OH-indolyl)	0.31	0.78	2500
	1f	CH_2-3-(5-OH-indolyl)	0.11	0.31	2800
	1g	$CH_2CONHCH_2Ph$	0.24	0.54	2300
	1h	(*R*)-CH_2Ph	0.31	0.29	940
	1i	CH_3	0.91	0.41	450
	**2a**	X = O	1.3	0.32	230
	2b	NCH_3	0.26	0.19	730
	**3a**	X = H, R = Me	2.7	0.60	220
	3b	X = OH, R = O-4-picolyl	0.081	0.12	1500
	3c	O-3-picolyl	0.078	0.21	2700
	3d	OCH_2CH_2Ph	0.061	0.093	1500
	3e	O-2-naphthyl	0.043	0.070	1600
	3f		0.087	0.38	1400
	**4**	R = CH_2-3-(5-OH-indolyl)	0.079	0.54	6800

tions below 100 μM. Similar findings have also been observed (data not shown) using the U87 glioblastoma cell line and the clinically important cytotoxic agent BCNU [20].

Pharmacokinetics, Pharmacodynamics, and Bioavailability of** 1b **in Mice

Preliminary experiments with mice showed that compound **1b** has sufficient solubility, stability, and bioavailability for further in vivo studies. Collected serum samples were extracted with ethyl acetate and the separated organic layer was analyzed using analytical HPLC or LC-MS. Serum levels were calculated using a calibration curve. When injected intraperitoneally, the concentration of **1b** in serum samples collected 5 min after injection was 11 and 24 μM for 20 and 40 mg/kg dosages, respectively. At the higher dose, the serum concentration dropped to 11 μM after 10 min, and below the lower limit of quantitation (3 μM) after 30 min. Together, these results demonstrate that compound **1b** can maintain a pharmacologically useful concentration in serum in a dose-dependent manner, and that it has a relatively short half-life in serum. No metabolites could be reliably detected.

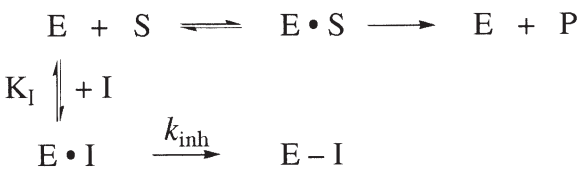


Figure 3. Enzyme Inhibition by an Irreversible Inhibitor

E, S, P, and I are enzyme, substrate, product, and inhibitor, respectively.

Our interest in pharmacologically useful TG2 inhibitors is especially motivated by their potential utility for treating Celiac Sprue, a widespread disorder of the small intestine for which no pharmacologic therapy is currently available (see for example [21–23]). For this application, an inhibitor with a short serum half-life is desirable so that the compound, when dosed orally, is primarily localized to the diseased organ (the small intestine). At the same time, the TG2 inhibitor must also have adequate bioavailability so that it can penetrate into the intestinal lamina propria, the primary location of pathologically relevant TG2 [24]. To estimate the oral bioavailability of **1b**, the compound was dosed orally in mice at 80 mg/kg. Blood samples were collected 30

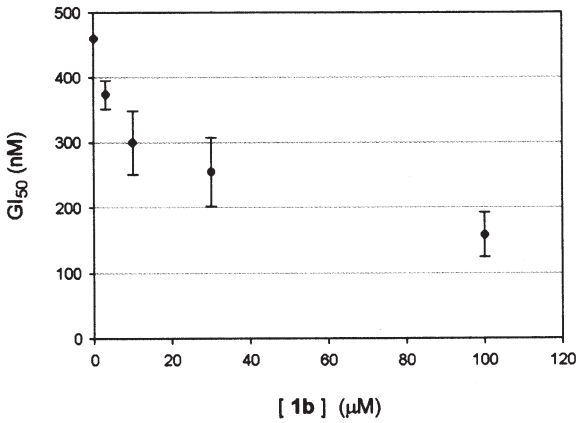


Figure 4. Effect of TG2 Inhibitor **1b** on GI_{50} for Epothilone C

The error bars represent data from assays performed four times with eight individual measurements for each condition.

min and 1 hr after oral gavage, and the concentration of **1b** was measured. At both time points, the observed serum concentrations were 5.5 μM and 4.7 μM, respectively, demonstrating good oral bioavailability of the compound.

The attractive bioavailability characteristics of compound **1b** prompted us to measure its inhibitory effect on TG2 in the small intestinal tissue. Mice were given a single oral dose of vehicle (40% DMSO), 40 mg/kg **1b**, or 60 mg/kg **1b**. After 4 hr the animals were sacrificed and specific TG2 activity was measured in proximal small intestinal tissue (the primary site of Celiac Sprue lesions in humans), as described in the Experimental Procedures. The average TG2 specific activities were 43 pmol/mg-min, 14 pmol/mg-min, and 8 pmol/mg-min (average of two mice), respectively, indicating that dose-dependent inhibition could be achieved in the small intestine.

Safety of** 1b **in Mice

To assess potential drug toxicity caused by long-term dosing, mice were injected daily with **1b** (i.p., 40 mg/kg) or vehicle (40% DMSO) for 14 days. All five inhibitor-treated mice survived the regimen without significant weight loss or gross toxicity. Serum samples were analyzed after the final dose using standard hepatic enzyme and bilirubin assays. Alkaline phosphatase (ALP) and bilirubin levels were not statistically different between the groups of mice treated with **1b** or vehicle only (ALP, 98 ± 42 versus 146 ± 9 U/l; bilirubin, 0.16 ± 0.11 versus 0.13 ± 0.07 mg/dl), whereas the level of alanine aminotransferase was elevated in the inhibitor-treated mice (360 ± 122 versus 79 ± 45 U/l). These results suggest that mice can tolerate relatively high systemic doses of a dihydroisoxazole inhibitor such as **1b** for at least 2 weeks. Elevated liver enzyme levels suggest that hepatic tissue is a potential primary site of the dose-limiting effects of compound **1b**.

Chemosensitization of Refractory Glioblastoma Tumors by** 1b **in Mice

Based on the good safety and in vitro efficacy of **1b**, we were prompted to evaluate its ability to enhance the sensitivity of chemotherapy against refractory tumors in animals. Balb/C mice were injected subcutaneously with 2×10^6 syngeneic DBT glioblastoma cells, and tumors were allowed to establish for 1 week [25–27]. Mice were then treated with daily intraperitoneal injections with either vehicle or TG2 inhibitor starting on day 8 for 10 treatments. On day 10 and 12, mice were coinjected with vehicle or BCNU (10 mg/kg and 5 mg/kg, respectively). This dosage schedule of BCNU results in minimal effect on the DBT tumors in this experimental paradigm. Tumors were removed and weighed 3 weeks after start of the experiment, and the mean tumor weight was separately calculated for each cohort. The results, summarized in Figure 5, demonstrate excellent dose-dependent efficacy of **1b**.

Visualizing the Action of Dihydroisoxazole Inhibitors on Cells

TG2 is found in various tissues and subcellular locations. To understand the biological and pathogenic roles of this ubiquitous enzyme, small molecule agents are needed to visualize its spatial distribution and temporal dynamics. Toward this goal a fluorescent (dansyl) dihydroisoxazole analog 5 was synthesized. The compound has absorption and emission maxima at 350 nm and 550 nm, respectively. Treatment of HCT-116 cells with 50 μM of analog 5 for 1 hr resulted in strong labeling of the cell plasma membrane, together with weaker but distinct granular labeling of the cytosol (Figure 6). The former pattern presumably reflects the abundance of TG2 anchored in the extracellular matrix of HCT-116 cells, whereas granular intracellular staining may be related to its presence in secretory granules or to a potential receptor-mediated endocytotic process.

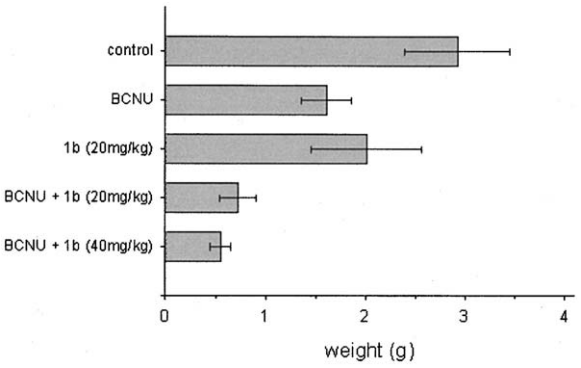


Figure 5. Weights of DBT Glioblastoma Xenografts after Treatment with BCNU Alone or in Combination with TG2 Inhibitor **1b**

The decrease in mean tumor weight in the groups treated with combination **1b** and BCNU was significantly different from both the control and the BCNU groups as assessed with a two-tailed Student's t test for independent variables. Significance was determined with $P < 0.05$.

Discussion

Human TG2 is a structurally and mechanistically complex protein [28] with multiple biological functions [1–3]. It is an especially interesting medicinal target, since it plays a crucial role in the pathogenesis of several unrelated disorders, yet it appears to be nonessential in mammals. Consequently, small molecule inhibitors of this enzyme can be useful probes not just for TG2 biology but also its role in a variety of diseases. Here we

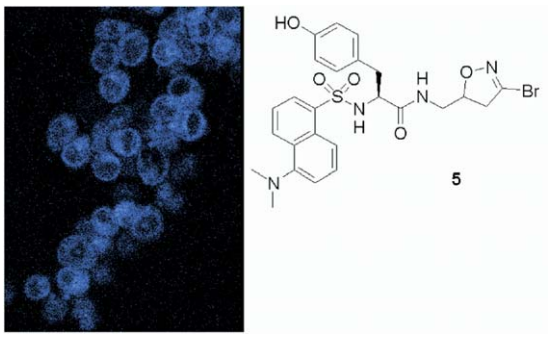


Figure 6. Two-Photon Confocal Microscopic Image of HCT-116 Cells Incubated with Fluorescent Dihydroisoxazole Compound **5**

$K_I = 0.11$ mM, $k_{inh} = 0.037$ min^{-1}, $k_{inh}/K_I = 340$ min^{-1}M^{-1}.

have analyzed the structure-activity relationships of dihydroisoxazole inhibitors of human TG2 and investigated the properties of a lead compound in vitro and in vivo.

A series of compounds with variations at three different positions have been prepared and tested to examine the relationship between the molecular structure and the inhibition properties. Based on their inhibitory activities, together with each compound's stability and solubility in aqueous solutions, one of the compounds was selected for further investigations into the biological properties of the dihydroisoxazole inhibitors. The pharmacological significance of TG2 in glioblastoma survival and resistance to chemotherapy has recently been highlighted (L.Y. et al., unpublished data). In vitro studies validated the hypothesis that TG2 inhibitors, while nontoxic by themselves, can sensitize tumor cells to modulation by other pharmacological agents. For example, tumor cell lines that are ordinarily refractory toward cytotoxic agents such as epothilone and BCNU can be sensitized by pretreatment with dihydroisoxazole inhibitors of TG2. Importantly, this chemo-sensitization ability of dihydroisoxazole **1b** was validated in mice harboring refractory glioblastoma tumors. Glioblastomas are notoriously resistant to standard clinical treatment protocols with radiation and chemotherapy. The mean survival in patients diagnosed with glioblastomas is about 12 months and has failed to improve over the years, despite modern neurosurgical procedures, sophisticated conformal radiation techniques, and multiple attempts with chemotherapy regimens. Given the acute need for defining new treatment strategies in glioblastoma patients, dihydroisoxazole inhibitors such as the compounds reported here could be used to evaluate the incremental benefit of blocking TG2 in conjunction with standard radiotherapeutic or chemotherapeutic protocols.

Our preliminary studies with dihydroisoxazole **1b** in mice have also highlighted several potentially attractive characteristics of this class of compounds for chronic disorders in which TG2 is believed to play a role. For example, Celiac Sprue is a widespread inflammatory disease characterized by malabsorption, abnormal small-intestinal structure, and intolerance to gluten, a complex mixture of nutritionally important proteins found in common dietary foodgrains such as wheat, rye, and barley [21–23]. Since gluten is an unlabeled ingredient in many packaged, bottled, and canned foods, a strict gluten-exclusion diet is very difficult to maintain and there is urgent need for therapeutic options. Although ex vivo studies with intestinal biopsy tissue from Celiac Sprue patients have demonstrated a critical role for TG2 in gluten-induced pathogenesis [29], the absence of an animal model for this widespread disease underscores the need for a suitable experimental therapeutic agent to validate TG2 as a therapeutic target for Celiac Sprue. In addition to potency and selectivity, such a small molecule would be orally bioavailable, have short serum half-life so as to minimize systemic distribution, and be well tolerated for the duration of a controlled, proof-of-principle clinical study (2–4 weeks). Our findings demonstrate that **1b** has many of these properties. Over a 2 week period of high-dose daily administration, it revealed a potential for hepatotoxicity based on elevation of serum transaminase levels without a significant effect on alkaline phosphatase or bilirubin. Further studies would be required to assess whether this is an intrinsic property of all TG2 inhibitors or whether it reflects non-mechanism-associated toxicity of **1b**.

Significance

We have investigated a series of dihydroisoxazole compounds for their potential inhibition activities against human TG2 and identified one of the compounds as a lead compound. In vivo experiments showed that this inhibitor is orally bioavailable, nontoxic, and active in animal models. This compound showed synergic effects to enhance chemosensitivity of tumor cells toward cytotoxic reagents. In addition to the potential therapeutic relevance of dihydroisoxazole inhibitors of human TG2, such compounds also offer the potential to provide fundamentally new and mechanistically fascinating insights into cell biology. A plethora of functions have been suggested for this multidomain protein in the context of the extracellular matrix, cytosol, and mitochondria; this is further complicated by the absence of a signal sequence encoded within the TG2 gene [1–4]. Most recent investigations into TG2 biology have relied upon the use of competitive substrate analogs such as cystamine and monodansyl cadaverine (see for examples [30–32]). By virtue of their dual advantage of specificity and spectroscopic characteristics, fluorescent dihydroisoxazole inhibitors such as 5 could be used to shed light on many unanswered questions about this most remarkable enzyme.

Experimental Procedures

TG2 Inhibition Assay

Recombinant human TG2 was expressed in *E. coli* and purified to >90% homogeneity as before [14]. Compounds were assayed in a reaction mixture containing 200 mM MOPS (pH = 7.2), 5 mM $CaCl_2$, 1 mM EDTA, 10 mM α-ketoglutarate, 18 U/ml glutamate dehydrogenase, 0.4 mM NADH, 3.3% (v/v) DMSO, 0.5 μM TG2, and 2, 5, or 10 mM Cbz-Gln-Gly·Na (K_M = 11 mM, k_{cat} = 37 min^{-1}). The enzyme reaction was started by addition of TG2 and the consumption of NADH was monitored by UV spectroscopy (340 nm, ϵ = 6220 $cm^{-1}M^{-1}$). Kinetic parameters were obtained by plotting the reaction progress curve against theoretical equations for irreversible enzyme inhibition [12].

HCT-116 Cell Growth Inhibition Assay

HCT-116 cells were grown in RPMI medium 1640 supplemented with 10% (vol/vol) FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. Cells were grown for 24 hr at 37°C and 5% CO_2 in air in a humidified incubator at 10,000 cells/well, and were then exposed to varying concentrations of **1b** (0–100 μM) for 24 hr. The media with inhibitor was then replaced with media containing epothilone C (0–4 μM) and incubated for an additional 24 hr. Viable cells were then measured using the MTT assay. For this, 10 μl of a 5 mg/ml MTT stock solution in PBS was added per 100 μl of media, and incubated with the cells for 2 hr at 37°C. The media and MTT solution were then removed by aspiration and the cells were solubilized in DMSO and transferred to a 96-well plate, and the absorbance was read at 570 nm by use of a Molecular Devices SpectraMax 190 96-well plate reader. The assay was performed four times with eight individual measurements for each condition.

Serum Testing

C3H mice (2–5 months old) were given the TG2 or carrier alone then euthanized by CO_2 inhalation. Blood was collected by intracardiac puncture followed by analysis of serum for alkaline phosphatase (U/l), total bilirubin (mg/dl), and alanine aminotransferase (U/l).

Measurement of TG2 Activity in the Small Intestine Tissue

Immediately after sacrificing each mouse, approximately 15–20 cm of intestine distal to the ligament of Treitz was excised, wrapped in parafilm, and immediately frozen on dry ice. The intestines were then transferred to a −80°C freezer for storage. Before thawing, they were weighed, diced into small pieces using a razor, and thoroughly rinsed with water using a squeeze bottle. They were then dounced in 50 mM mannitol, 2 mM Tris-HCl, 10 mM calcium chloride (pH = 7.1) at 4°C to give a 20% homogenate.

The incorporation of [1,4^{14}C]-putrescine (Amersham Biosciences) into N,N-dimethyl casein (Sigma-Aldrich Co.) was based upon a modification [33] to the filter paper method of Lorand [34]. The assay was initiated by adding 80 μl homogenate to 320 μl substrate buffer (83 mM dithiothreitol, 83 mM Tris-HCl, 16.7 mM calcium chloride, 4.17 mg/ml N,N-dimethyl casein [pH = 9.0], and 0.167% Triton X-100) prewarmed to 37°C. The reaction mixture was then incubated at 37°C, and 30 μl was spotted on dry 3-MM Whatman chromatography paper (1 in × 1 in) presoaked in a 10% solution of trichloroacetic acid (TCA) and immediately plunged into 10% TCA to precipitate the protein. Each filter paper was soaked 15 min in 10% TCA, 15 min in 5% TCA two times, 3 min in 50% ethanol/50% acetone, and 2 min in acetone. The filter papers were air dried, placed in 5 ml Ready Safe Scintillation Cocktail (Beckman Coulter), and counted on a Beckman LS 3801 liquid scintillation counter. Time points were taken every 15 min for an hour and the reaction rate was calculated using linear regression. Each point was measured in duplicate, and nonwashed samples were used to quantify the amount of putrescine incorporated. Protein concentrations were determined using the Bio-Rad protein assay.

Glioblastoma Mouse Tumor Model

Detailed procedures for these studies are described elsewhere [L.Y. et al., unpublished data].

Supplemental Data

Synthesis and characterization of compounds **1–5** is available online at http://www.chembiol.com/cgi/content/full/12/4/469/DC1/.

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