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Abstract: In recent years there has been a clear consensus that neurodegenerative conditions can be better treated through concurrent modulation of different targets. Herein we report that combined inhibition of Transglutaminase 2 (TG2) and Histone Deacetylase (HDAC) protects synergistically against toxic stimuli mediated by glutamate. Based on these findings, we designed and synthesized a series of dual TG2-HDAC binding agents effective at low micromolar concentrations.

Transglutaminases (TGs) and HDACs are a family of enzymes implicated in neurodegeneration. In particular, their inhibition showed a protective effect in several neurodegenerative conditions possibly because they were both shown to be induced in the presence of toxic stimuli and to be easily modulated by pharmacological and molecular intervention. TG2, the most studied and expressed isoform of the TG family, has been linked to neurodegeneration since the observation that it might be important in cross-linking proteins in Alzheimer's disease.[1] Later, it was reported that the genetic elimination of TG2 in Huntington's disease (HD) mice models ameliorated the symptoms[2] and restored the message and protein levels of brain-derived neurotrophic factor (BDNF), a neurotrophin involved in neuronal development, vitality and synaptic plasticity.[8],[4] Furthermore, TG2 was observed at the promoters of repressed genes in HD and microarray analysis showed that TG2 inhibition corrected transcriptional dysregulation in HD and rescued the eye phenotype in a *Drosophila* model of polyglutamine diseases.[5]

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Accordingly, their inhibition or genetic suppression halted oxidative stress-induced cell loss in cortical neurons.[6]

HDAC inhibitors (HDACIs) had a protective effect in several *in vivo* models of neurodegenerative diseases.[7] For instance, HDACIs induced the expression of BDNF[8], and protected cultured cortical neurons against oxidative stress induced by glutathione-depletion[9] and excitotoxicity induced by glutamate.[10] Furthermore, Vorinostat (SAHA), a well-known HDACI, was reported to enhance memory in animal models.[11]

Of note, previous work showed that a combinatorial treatment of a *Drosophila* model of HD with SAHA and cystamine, a broad TG inhibitor, was protective[12], but the low stability and deliverability of cystamine in the central nervous system (CNS) limited its administration *in vivo*.[13]

Based on these premises, herein, we report the synergistic neuroprotective effects of TG2 and HDAC inhibition in an *in vitro* model of neuronal oxidative stress and describe the design, synthesis and preliminary investigations of a new class of hybrid compounds able to inhibit TG2 *and* HDAC and to halt toxicity induced by glutathione deprivation.

In order to identify a drug combination that would synergistically protect neuronal cells from oxidative stress, we examined the effects of a TG2 inhibitor, namely B001, and a HDAC inhibitor, namely sodium butyrate, either alone or in combination (Figure 1). As shown in Figure 1A, the compounds do not induce any toxic effect at the tested concentrations (B001 at 5 μM, sodium butyrate at 250 μM) on immature neurons. Surprisingly, co-treatment with B001 and sodium butyrate synergistically protected cells against glutamate at the tested concentrations (glutamate alone, 28% viable cells; glutamate and B001, 37.5% alive cells; sodium butyrate, 44.3% alive cells; glutamate, B001 and sodium butyrate, 67.5% alive cells;). To determine whether the synergy observed was general or inhibitor-specific, we performed combination experiments with other TG2 and HDAC inhibitors, such as cystamine and SAHA. When the neurons were treated with non-protective doses of cystamine (500 nM) and SAHA (500 nM), independently, both compounds failed to rescue neurons from neuronal death, showing 28% and 36.7% of alive cells, while neurons treated only with glutamate showed a 22% viability (Figure 1B). Notably, when the drugs were administered in combination, the number of viable cells increased to 59.8%, representing a significant increment of 37.8% in cell survival.

These results opened the possibility to combinatorial drug therapy for TG2 and HDAC inhibition. However, combinatorial therapy suffers from several drawbacks and, to overcome these problems, the multi target approach has emerged in drug discovery in the last decade.[14]

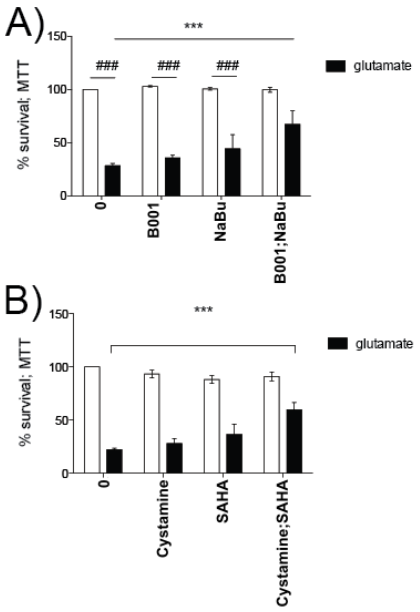


Figure 1. Combinatorial treatment with TG2 and HDAC inhibitors protects neurons against oxidative stress. A) B001 (5 μM) and sodium butyrate (NaBu; 500 μM) where administered alone or in combination for 16 h in the presence of absence of glutamate (5 mM). ### p<0.001 glutamate treated versus untreated; ***p<0.001 glutamate treated versus glutamate plus B001 and NaBu. B) Cystamine (500 nM) and SAHA (500 nM) where administered alone or in combination for 16 h in the presence of absence of glutamate (5 mM). ### p<0.001 glutamate treated versus untreated; ***p<0.001 glutamate treated versus glutamate plus B001 and SAHA. Two-way ANOVA, *Bonferroni's* post hoc test.

Therefore, we wondered whether it might be of any advantage to design a class of Multiple Ligands (ML) able to modulate both targets instead of using different inhibitors. HDACI-based MLs have found massive application in the field of anticancer drug discovery while only recently Oyarzabal and coworkers have reported the first HDAC-phosphodiesterase dual inhibitors as neuroprotective agents.[15]

Concerning TG2 inhibitors, only a few classes of inhibitors have been discovered;[16] among them, of particular note are the 3-(substituted cinnamoyl)pyridines, such as compound **1** (Figure 2).[17] By analyzing the structure-activity relationships of this series of compounds, it turned out that the *para* position of the cinnamoyl phenyl ring could be substituted with bulky groups without reduction of the inhibitory potency. Notably, to the best of our knowledge, TG2 inhibitors have never been used before to design MLs.

Different strategies could be adopted to design MLs. In this case, we coupled the structures of SAHA, a well-known HDACI, and **1** in order to obtain hybrid compounds that could interact with both HDAC and TG2 (Figure 2). Compounds **2-4** are characterized by linkers of a different length between the hydroxamic acid and the phenyl ring. Unfortunately, the compound bearing a three-methylene spacer was synthetically inaccessible.

For the synthesis of target compounds **2-4**, a straightforward convergent approach was developed, starting from available compounds **5**[17] and **7-9**[18] (Scheme 1). The primary amino group of compound **5** was converted into the corresponding isothiocyanate by reacting with 1,1'-thiocarbonyldi-2(1H)-pyridone affording the compound **6**. Trityl-protected amino hydroxamates **10-12** were obtained by a reduction of the azido group of the trityl-protected azido hydroxamates **7-9**.

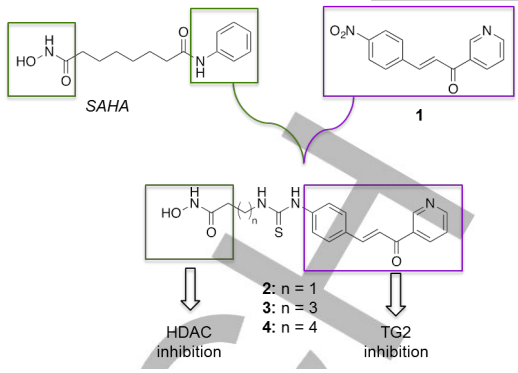
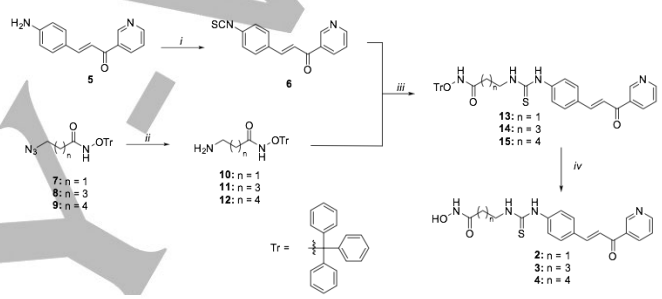


Figure 2. Design strategy leading to hybrid compounds **2-4**.

Coupling of compound **6** with amines **10-12** led to the thiourea-linked adducts **13-15**. Final deprotection of the hydroxamic acid, in presence of triethylsilane and trifluoroacetic acid, led to the desired compounds **2-4**.



Scheme 1. *i*) 1. 1,1′-Thiocarbonyldi-2(1*H*)-pyridone, DMF, rt, 24h, 72% yield; *ii*) PPh_3, $MeOH/H_2O$, rt, overnight, 54-79% yields; *iii*) Et_3N, DMF, rt, 24h, 59-81% yields; *iv*) TFA, Et_3SiH, DCM, rt, 44-79% yields.

Compounds **2-4** were evaluated for their ability to inhibit TG2 and HDAC1 (Table 1). Concerning TG2 inhibition, the most active compound is **3**, characterized by a four methylene spacer, with an IC_{50} of 13.3 ± 1.5 μM (K_i^{app} = 10.5 μM), followed by compound **4** and **2** which turned out to be equally active. These excellent IC_{50} values clearly indicate that the introduction of the hydroxamic acid does not have a detrimental effect on the binding of the cinnamoyl-pyridines to TG2[17].

Table 1. Effects of compounds **2-4** and SAHA on human TG2 and HDAC1 activity.

Compounds	n	TG2		HDAC1 % residual activity at 5 μM
		IC_{50} (μM)[a]	K_i^{app} (μM) (α=1.27)c [b]	
1		21 ± 4[c]	5.6[c]	n.d.[d]
2	1	24.3 ± 1.2	19.1	58.00 ± 6.46
3	3	13.3 ± 1.5	10.5	15.58 ± 2.22
4	4	22.1 ± 2.0	17.4	11.52 ± 0.90
SAHA		n.d.[d]	n.d.[d]	27.74 ± 6.38

[a] IC_{50} values are defined as the drug concentration that reduces by 50% the activity. [b] K_i^{app} was calculated according to the Cheng-Prusoff equation[19] [c] Determined with guinea pig liver TG2[17]. [d] n.d.: not determined.

Compounds **2-4** were then evaluated for their inhibitory activity towards HDAC1; Table 1 reports percentage of residual HDAC1

activity for compounds **2-4** at 5 μM compared to SAHA. Compounds **3** and **4** show similar inhibitory activity, and turned out to be slightly more active than SAHA. This is not surprising since it is well known that the HDAC is able to accommodate a vast array of different chemical structures within its active site.

In order to evaluate whether the *in vitro* inhibitory activities translated into intracellular inhibition of both enzymes, compound **3** was further evaluated in cell-based assay. In particular, western blotting analyses were performed using human neuroblastoma SH-SY5Y cell line to determine the effects of compound **3** on acetylation levels of tubulin and histone H3 at lysines 9/14 (H3K9K14ac) (Figure 3A).

Table 2. IC_{50} Values (μM) of Compound **2-4** against the HDAC1 and 6 isoforms

Compounds	n	IC_{50} vs HDAC (μM)	
		HDAC1	HDAC6
2	1	4.0	17.00
3	3	3.38	4.11
4	4	8.50	4.75
SAHA		0.6[j]	5.6

[a] IC_{50} values are defined as the drug concentration which reduces by 50% the activity. [b] K_i^{app} was calculated according to the Cheng-Prusoff equation[19] [c] Determined with guinea pig liver TG2[17]. [d] n.d.: not determined.

Cells were treated with compound **3** and its parent compounds, SAHA (5 μM) and **1**, for 30 h at three different concentrations (0.1, 1 and 5 μM). From Figure 4 it can be observed that compound **3** is able to induce hyperacetylation of tubulin, compared to the control, although in a lesser extent compared to SAHA and such effects are already visible at the concentration of 0.1 μM, and it is dependent on the concentration used. Concerning acetylation of histone H3, compound **3** is not able to induce any increase of acetylated H3 compared to control. This effect is in contrast with the observation that SAHA induces a marked increase of acetylated H3. Since tubulin is a substrate of HDAC6 and acetylated tubulin levels function as a biochemical marker for HDAC6 cellular activity, the increase in the level of acetyl-α-tubulin compared to H3K9K14ac could be due to an intrinsic selectivity of compound **3** for HDAC6. The ability of compound **3** to inhibit the transamidase activity of TG2 was further tested by incubating recombinant purified human TG2 with HEK293T cell lysate in the presence or absence of the inhibitor. A biotinylated peptide, known to be a TG2 substrate, was added to the protein lysate and the efficiency of TG2 in crosslinking the biotinylated peptide to proteins was evaluated by streptavidin blotting. As shown in Figure 3B, compound **3** significantly reduced (by 33%) the activity of 1 μg of TG2 when used at final concentration of 50 μM.

To confirm our previous data on the synergistic effect of HDAC and TG2 inhibition, we treated cortical neurons with compound **3** and tested its ability to halt neuronal death. Cells were treated with increasing concentrations of compound **3** in the absence of toxic stimuli. In Figure 4A, compound **3** does not exert any toxic effects up to the highest concentration tested (50 μM). Then, we evaluated its protective effect in the presence of glutamate (5 mM) (Figure 4A and B). We observed that compound **3** protects neurons already at the concentration of 6.3 μM where ~80% of the neurons are preserved, and at higher concentrations, a complete protective effect is achieved (at 25 μM 95% of alive cells, at 50 μM 100% alive cells). The EC_{50} calculated for compound **3** under these experimental conditions was 3.7 ± 0.5 μM.

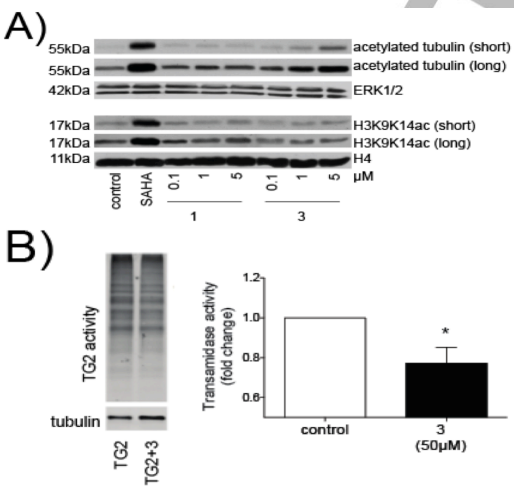


Figure 3. A) Western blot probing for acetylated tubulin and histone H3K9K14ac in the SH-SY5Y cell line after 30 h treatment with SAHA and compounds **1** and **3**; B) Streptavidin blotting showing a decreased TG2 activity in the presence of compound **3** (50 μM). The gel image is representative of **3** and the graph shows normalized fold change in streptavidin reactivity ± SEM. *p<0.05; Student t test.

In summary, we report the development of a new class of MLs able to modulate both targets. In particular, compound **3** inhibits both HDAC and TG2 either *in vitro* and cells, exerts neuroprotective activity at low micromolar concentrations and does not show any toxic effects up to 50 μM. The present findings open up a valuable strategy to discovery new promising neuroprotective molecules.

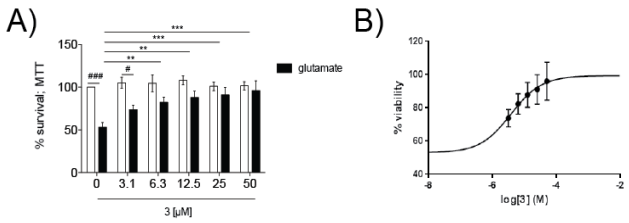


Figure 4. A) Compound **3** halts oxidative stress-mediated cell death. Analysis of cell viability in mouse primary cortical neurons treated with glutamate (5 mM) and compound **3** at different concentrations. Graph, mean ± SEM; n=3; **p<0.01, ***p<0.001, comparing glutamate-treated and compound **3** plus glutamate-treated neurons. ##p<0.01 and ###p<0.001 comparing plus and minus glutamate, Two-Way ANOVA and Bonferroni's post hoc test. B) Cell viability was measured as a function of the concentration of compound **3**; data points are mean ± SEM, n=4. The solid line derives from fitting to a sigmoidal curve (see Experimental section), providing a value of EC_{50} = 3.7 ± 0.5 μM.

Acknowledgements

This work was supported by: University of Bologna (RFO), The Alzheimer Trento Onlus with the legato Baldrachi, EU, Blueprint project no. 282510; the Italian Flag Project EPIGEN; PRIN-

20152TE5PK_003; AIRC (contract n°17217), and P.O.R. Campania FESR 2007-2013-MOVIE (B25C13000240007). K.Y.P.A. is grateful to the Fonds de Recherche Québécois: Nature et Technologies (FRQNT) for a doctoral scholarship.

Keywords: neurodegeneration • transglutaminase 2 • histone deacetylase • hydrid compounds • multiple ligands

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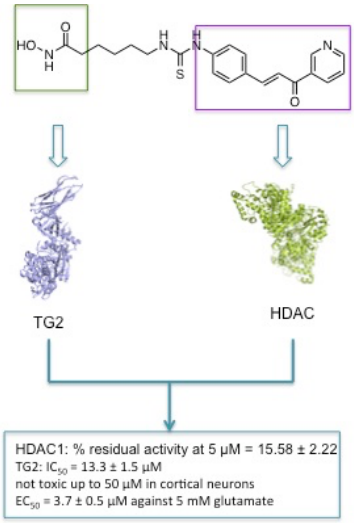

We discovered that concomitant inhibition of HDAC and TG2 synergistically protects cortical neurons from toxic stimuli induced by glutamate. Based on this finding, we have developed a new class of multiple ligands able to modulate both targets. Compound **3** inhibits both targets either in vitro and in-cells and is effective in halting oxidative stress-mediated neuronal death.