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Targeting Botulinum A Cellular Toxicity: A Prodrug Approach

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Abstract

The botulinum neurotoxin light chain (LC) protease has become an important therapeutic target for post-exposure treatment of botulism. Hydroxamic acid based small molecules have proven to be potent inhibitors of LC/A with nanomolar K_i values, yet, they lack cellular activity conceivably due to low membrane permeability. To overcome this potential liability, we investigated two prodrug strategies, 1,4,2-dioxazole and carbamate, based on our 1- adamantylacetohydroxamic acid scaffold. The 1,4,2-dioxazole prodrug did not demonstrate cellular activity, however, carbamates exhibited cellular potency with the most active compound displaying an EC_{50} value of 20 μ M. Cellular trafficking studies were conducted using a “fluorescently silent” prodrug that remained in this state until cellular uptake was complete, which allowed for visualization of the drug’s release inside neuronal cells. In sum, this research sets the stage for future studies leveraging the specific targeting and delivery of these prodrugs, as well as other antbotulinum agents, into neuronal cells.

INTRODUCTION

Botulinum neurotoxins (BoNTs), produced and secreted by the anaerobic spore-forming Gram-positive bacillus *Clostridium botulinum*, are the most poisonous biological toxins known^{1, 2} and thus have been classified by the Centers for Disease Control and Prevention as a “Category A” agent placing them among the top six highest risk agents for bioterrorism. Ingestion or inhalation of BoNT causes the paralytic illness botulism characterized by peripheral neuromuscular blockade and progressive flaccid paralysis.³ Seven serologically distinct BoNT serotypes (A-G) have been identified; serotype A (BoNT/A) is the deadliest and most life-threatening with a lethal dose for humans of ~1 ng/kg of body weight accompanied by a prolonged half-life *in vivo*.⁴ This extreme potency along with the neurospecificity and long persistence of BoNT/A in neurons has alternatively made this toxin an effective therapeutic agent for the treatment of a vast array of human disorders and conditions, such as dystonia, tremors, migraines, and even facial wrinkles.^{5, 6}

BoNT is synthesized as an ~150 kDa single polypeptide chain that undergoes post-translational proteolysis to form the mature protein consisting of a 100 kDa heavy chain

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Supporting Information. ¹H NMR and ¹³C NMR spectral information and laser scanning confocal microscopy figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

(HC) domain and a 50 kDa light chain (LC) domain bound by one or more disulfide linkages.^{7, 8} The HC is responsible for neurospecific binding, receptor-mediated endocytosis, and pH-dependent translocation of the LC into the cytosol of the cell. The LC is a zinc-dependent endopeptidase that catalyzes the cleavage of SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins SNAP-25, VAMP/synaptobrevin, and syntaxin, which mediate cellular and vesicle fusion leading to the release of acetylcholine into the neuromuscular junction.^{7, 8} Cleavage of SNARE proteins by the LC results in the loss of neurotransmission causing neuromuscular paralysis and in severe cases may lead to respiratory failure, cardiac arrest, and ultimately death.

The potential use of BoNT as a bioweapon or misuse in a clinical setting has fueled the search for an effective treatment against unwarranted and possibly fatal BoNT exposure. This is especially pertinent as cellular intoxication occurs rapidly and symptoms of botulinum poisoning can appear within 24 hours or less.³ Current countermeasures that rely solely on the passive administration of antibodies quickly become ineffective due to their inability to combat BoNT once it binds and begins to undergo endocytosis into the cell.⁹ Therapies that target BoNT intracellularly could overcome antibody sequestering liabilities and prove to be an important post-exposure treatment for BoNT intoxication.

The BoNT LC/A has been the primary focus of therapeutic intracellular efforts due to this serotype's extreme potency and long lasting paralysis. In particular, many research groups have examined small molecule inhibitors targeting its protease activity,^{9, 10} including competitive LC/A inhibitors,^{11–17} as well as, covalent^{18–20} and exosite.^{21, 22} Our laboratory has identified a collection of hydroxamic acid inhibitors that have proven to be some of the most active compounds reported to date, with nanomolar K_i values (Figure 1).^{23–26} Unfortunately, two of the most promising compounds, **1** and **4** (Figure 1), were found to be cytotoxic when evaluated in cell-based assays. Interestingly, 1-adamantylacetohydroxamic acid **3** (Figure 1), a simpler homologue of **4**, was not cytotoxic even at a concentration of 250 μ M in human induced pluripotent stem cells (hiPSC) derived neurons.²⁷ Encouraged by the potent LC/A inhibition by **3**, (K_i = 460 nM), along with the lack of cytotoxicity, we further investigated **3**'s BoNT/A cellular inhibitory activity in hiPSC-derived neurons. Disappointingly, this compound did not provide any protection against BoNT/A induced SNAP-25 cleavage, the intracellular substrate for LC/A. Based on these results, we hypothesized that a lack of cellular activity of **3** might be due to the inability of this compound to readily cross the cell membrane and therefore properly target BoNT LC/A once the enzyme has been translocated into the cytosol of the cell.²⁸ Yet, this assumption was viewed with trepidation as seemingly similar molecules such as amantadine and rimantadine (known antiviral drugs) are also based upon the adamantane scaffold (Figure 2).^{29, 30} Additionally, hydroxamates, such as suberoylanilide hydroxamic acid (SAHA), are effective anti-cancer agents (Figure 2).^{31, 32} However, even with this body of knowledge, membrane permeability appeared to be the most logical hindrance for a lack of cellular activity. Hydroxamic acids are known to undergo deprotonation due to their acidic nature ($pK_a \sim 8.0$ – 8.8)^{33, 34}, which could prevent diffusion through a cell membrane.

Therefore, a prodrug approach was undertaken with **3** in the attempt to increase membrane permeability by masking the polarity and ionizability of the hydroxamic functional group with a hydrophobic moiety. In contemplating such an approach we note examples of hydroxamic acid prodrugs that have been reported including 1,4,2-dioxazoles,³⁵ carbamates,²⁸ *O*-glucuronides and/or *O*-galactosides,³⁶ Mannich-based hydroxamic acid prodrugs,³⁷ and *O*-acylated hydroxamates.³⁸ Our initial prodrug investigations focused on 1,4,2-dioxazole and carbamate analogs of **3** as such strategies have been previously pursued for matrix metalloproteinase³⁵ and histone deacetylase²⁸ inhibitors, respectively.

RESULTS

CHEMISTRY

Thus, two prodrug strategies (*vide supra*) were considered to address what we believed to be a liability of the hydroxamic acid embedded within **3**. The first approach was 1,4,2-dioxazole **5**, which was readily prepared by reacting hydroxamic acid **3** with 2,2-dimethoxypropane via acid catalysis (Scheme 1, reaction a). As a second prodrug strategy, carbamoyl hydroxamate **6a** was viewed as an additional logical entry to increase the hydroxamate's cell permeability. In our initial attempt to prepare **6a**, hydroxamate **3** was reacted with benzylisocyanate. Unfortunately, this reaction while yielding the desired **6a**, also produced its isomer **7** in a ratio of approximately 1:3.5 as confirmed by ¹H/¹⁵N HSQC NMR (Scheme 1, reaction b). Due to the poor selectivity of the aforementioned reaction (15–20% of the desired *O*-acylated product **6a**), an alternative method was investigated. We found that the desired product could be synthesized by nucleophilic addition of benzylamine to 1,4,2-dioxazol-5-one, **8** (Scheme 2).³⁹ The reaction was indeed completely *O*-selective, affording **6a** as the sole product in high yield (88%). Encouraged by this result, intermediate **8** was reacted with a variety of amines to provide carbamate prodrugs **6a–l** (Scheme 2). Our choices here were based on the overall aim of improving cellular activity, yet, we also hoped these congeners might possess improved stability (*vide infra*).

Among the amines employed for the reaction with intermediate **8**, 7-amino-4-methylcoumarin (AMC) showed no reactivity, presumably due to the weak nucleophilicity of the amine moiety. Therefore, **6m** was prepared by a two-step procedure (Scheme 3). AMC was first treated with diphosgene to form a reactive intermediate (most likely a carbamoyl chloride), which was then reacted with hydroxamate **3** to furnish the desired carbamate product. The structure of **6m** was again confirmed by ¹H/¹⁵N HSQC NMR. It is worth noting that in the case of less reactive/nucleophilic anilines, we observed urea-type byproducts, presumably derived from **8** via the Lossen rearrangement, formation of which took place over prolonged periods of time. These observations (urea formation and limited stability of intermediate **8**) are in agreement with previous reports.^{39, 40}

Stability of the prepared prodrugs in PBS, pH 7.4 and culture medium was examined by LC/MS analysis (positive single ion monitoring (SIM) mode) in the presence of stable isotopically labeled (SIL) internal standards; except for fluorogenic prodrug **6m**, where fluorescence increase due to the release of free AMC was monitored.

Each prodrug was dissolved in buffer or culture media, and at timed intervals an aliquot was withdrawn. After the addition of an internal standard, the sample was analyzed by LC/MS to determine the amount of prodrug and released drug in the solution (see experimental section for details). Deuterium-labeled analogs of both the 1,4,2-dioxazole and carbamate prodrugs were synthesized to serve as the SIL internal standards (Scheme 4).⁴¹ Deuterated **5'** was prepared in an analogous fashion to compound **5**, using acetone-*d*₆ to introduce the deuterium label (Scheme 4, reaction a). The hydroxamate carbamate-based SIL internal standards were synthesized as described for prodrugs **6a–l** using the deuterated counterpart of the adamantane hydroxamate **3**. The deuterated compound **3'** was prepared in the following two steps: 1) H/D-exchange of the α-hydrogens of carboxylic acid **10** using benzene-*d*₆ in the presence of AlCl₃; 2) conventional coupling of acid **10'** with hydroxylamine (Scheme 4). The deuterium incorporation of **3'** was over 95% as determined by NMR. Due to the acidity of this α-position, potential loss of deuterium was investigated in PBS buffer, pH 7.4 at rt. LC/MS analysis showed that the deuterium content remained virtually unchanged for at least 5 days, which confirms the validity of our stability study.

BIOLOGICAL RESULTS

Cell-based assays provide an excellent model system that requires all of the steps necessary for BoNT/A induced toxicity, including cell receptor binding, endocytosis, translocation of the LC/A protease into the cytosol, and ultimately cleavage of SNAP-25 inside the cell. Therefore, the potency of LC/A inhibitors within neuronal cells can be determined by monitoring the compound's ability to protect against BoNT/A induced SNAP-25 cleavage. As an initial screen, compounds **5**, **6a** and **7** were tested at a concentration of 25 μ M in hiPSC-derived neurons.^{42, 27} The neurons were first exposed to BoNT/A (250 LD₅₀ Units) for 10 min followed by the removal of any remaining extracellular toxin prior to the addition of compound. Next, the cells were incubated for an additional 8 h in the presence of compound before quantifying the amount of cleaved and uncleaved SNAP-25 by Western blot analysis. It is important to note that this type of post-intoxication scenario is most relevant because it relies solely on the intracellular inhibition of LC/A,^{23, 43} in contrast to assays where toxin and compound are preincubated prior to their addition to cells or where toxin and compound are simultaneously added to the cells. Unfortunately, dioxazole **5** did not provide any protection against SNAP-25 cleavage; however, benzyl carbamate **6a** exhibited ~50% inhibition (Figure 3). Interestingly, **7** proved to be cytotoxic when tested at this concentration. The inactivity of prodrug **5**, as compared to **6a**, may be due to inefficient release of the parent drug **3** within the cell, which we posit could be enzyme-controlled. Importantly, our studies with recombinant BoNT LC/A demonstrate that prodrug **6a** is not a substrate for this enzyme (1–425 or 1–448), therefore the hydrolytic release of the active compound **3** within the neuronal cell would by default involve an enzyme other than the botulinum neurotoxin protease.

Prodrug **6a** was the only one of the three initial compounds tested that demonstrated promise in the cell-based assay and, therefore, was investigated further. The cellular potency, i.e. EC₅₀, value was determined as described above using varying concentrations of **6a**. As illustrated in Figure 4A, **6a** displayed a dose-dependent inhibition pattern with an EC₅₀ value of 20 μ M. Recently, Shoichet et al. described how colloidal aggregation can diminish the efficacy of drugs in cell-based assays.⁴⁴ While the free drug can diffuse through the cell membrane, drug sequestered in colloidal aggregates cannot, thereby decreasing the effective concentration within the cell. Due to the lipophilic nature of prodrug **6a**, we investigated whether a similar colloidal effect would be observed in our cell-based assay. Therefore, **6a** was tested in the absence or presence of 0.01% Tween-80, the highest concentration of detergent that had no effect on cell viability. As shown in Figure 4B, there was no impact on the EC₅₀ value, providing evidence that the compound exists as a free monomer under cell culture conditions. Lastly, the activity of prodrug **6a** against purified LC/A was evaluated in vitro.⁴⁵ The carbamate prodrug **6a** of parent hydroxamate **3** did not show any inhibition. This was not unexpected, as we speculated that release of the active compound **3** inside the cell would be required for both efficient targeting/inhibition of intracellular LC/A.

Encouraged by the cellular activity of benzylcarbamate **6a**, a series of carbamate-based prodrugs **6b–6l** (Scheme 2) were evaluated in hiPSC-derived neurons in the same manner, *vide supra*. Considering the EC₅₀ (20 μ M) of **6a** as a benchmark, we looked for improvement among this diversified series of compounds. Unfortunately, no increase in cellular activity was observed with respect to **6a** (Table 1). Thus, the fluorobenzyl carbamate **6c** showed similar activity with an EC₅₀ value of 27 μ M, (Table 1, entry 4), however, addition of the electron withdrawing group did not enhance its cellular activity as predicted. Compounds **6b** and **6f** displayed ~2-fold higher EC₅₀ values of 42 and 37 μ M, respectively (Table 1, entries 3 and 7). Unfortunately, the additional carbamates prepared, (Table 1, entries 5 and 8–13) were significantly less potent than **6a**. Based on these results, a benzylic substituent seems to be important for the cellular activity. We speculate that this can be

attributed to a suitable combination of hydrophobicity, stability, and/or enzymatic recognition by a yet to be identified hydrolase.

In trying to gain insights into these carbamoyl hydroxamates that now demonstrated cellular efficiency, we turned to the coumarin derivative **6m** (Scheme 3), which has a half-life of ~4.5 h in culture medium and displayed modest inhibition in our cell-based assay.⁴⁷ This analog was designed as a fluorogenic prodrug to surveil potential cellular trafficking inside the cell. In this vein, Burnett et al. have taken advantage of the inherent autofluorescence of some of their small molecule LC/A inhibitors and were able to establish entry into cells.¹⁴ In contrast, our strategy is based on the concept that **6m** is “fluorescently silent” until cleavage of the carbamate protecting group, wherein the highly fluorescent AMC would be released and thus visualized by fluorescence microscopy, emitting in the blue spectral region. Hydroxamic acid release of **3** from **6m** is directly linked with AMC formation, therefore uptake and cleavage of the carbamate-protected prodrug inside the cell can be observed.

Accordingly, prodrug **6m** was evaluated in neuronal cells (Neuro-2a) using laser scanning confocal microscopy (Figure 5). Cells were incubated with or without **6m** followed by counterstaining with wheat germ agglutinin a biochemical marker that stains the plasma membrane and Golgi, and TOTO-3, a nucleus stain. Upon optical excitation, the released AMC fluorophore is visibly distributed throughout a large number of the **6m** treated Neuro-2a cells (Figure 5, A1 and A2; see also Figure S1). Further analysis demonstrated that a portion of the AMC is colocalized with the Golgi, an intracellular organelle, confirming the presence of AMC within the neuronal cell. Importantly, no cellular uptake was observed when cells were incubated with free AMC alone (Figure S2) indicating that **6m** must first be taken up by the cell before the intracellular release of AMC, i.e., there is no passive transport of extracellular AMC across the cellular membrane.

CONCLUSIONS

Potent small molecule LC/A inhibitor 1-adamantylacetohydroxamic acid **3** was examined in cell-based assays and provided no protection against BoNT/A induced SNAP-25 cleavage in neuronal cells. The discrepancy between its sub-micromolar activity against the purified BoNT/A protease and lack of activity in cells led us to explore this compound further.²⁵ We hypothesized that one possible explanation could be low cellular uptake of hydroxamic acid **3**. To explore this hypothesis, we prepared one dioxazole-based (**5**) and twelve carbamate-like (**6a–6l**) prodrugs based on **3**. All of these hydroxamate prodrugs were tested in hiPSC derived neurons for their ability to inhibit intracellular SNAP-25 cleavage and EC₅₀ values were calculated accordingly (Table 1). Among this series of prodrugs, benzylcarbamoyl hydroxamate **6a** displayed the best activity in cells (EC₅₀ = 20 μM). The stability of **6a** in buffer or culture medium was acceptable, while its logP was modest (Table 1, entry 2). On the other hand, derivatization of the benzyl protecting group had little impact on logP, negatively affected inhibitory potency, and slightly lowered stability in buffer (Table 1, entries 3 and 4). Derivatives **6d–6h** showed an improved logP (except **6e**) as well as stability in solution, but the activity was considerably poorer with exception of prodrug **6f**, where the EC₅₀ value was approximately doubled (Table 1, entries 5–9). Unfortunately, phenylcarbamoyl hydroxamates (**6i–6l**) were unstable in buffer (t_{1/2} ~1 h), which undoubtedly was linked to their lack of cellular activity with estimated EC₅₀ values >100 μM (Table 1, entries 10–13). Coumarin **6m** was successfully utilized for visualization of cellular uptake and drug release inside neuronal cells allowing the intracellular behavior of the BoNT protease inhibitor in a neuronal cell to be tracked (Figure 5). The importance of this latter finding is that it establishes a framework for both spacial as well as temporal monitoring of potential drug-botulinum intracellular activity.

Finally, we make note that for **6a** to possess intracellular activity it must be hydrolyzed in the neuronal cell, most likely enzymatically based on its stability in cell culture medium and the time frame in which the BoNT/A assay is conducted. One of the major challenges set down by the botulinum neurotoxin community in realizing post-exposure therapies is the selective targeting and delivery of the countermeasure to the neuronal cell. In the future, it will be of high interest to see if hydrolysis of **6a** is linked to a specific neuronal intracellular enzyme, which if realized could be explored for the specific unmasking of BoNT prodrugs.

EXPERIMENTAL SECTION

Chemistry

All reagents were purchased and used as received unless otherwise stated. Reactions were carried out under a nitrogen atmosphere with dry, freshly distilled solvents under anhydrous conditions, unless otherwise noted. Methylene chloride (CH_2Cl_2) was distilled from calcium hydride. Yields refer to chromatographically and spectroscopically homogeneous materials, unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25-mm EMD silica gel plates (60F-254) using KMnO_4 staining or UV-light (254 nm). Flash chromatography separations were performed on Silicycle silica gel (40–63 mesh). All compounds were established to be 95% pure using HPLC. NMR spectra were recorded on Bruker spectrometers and calibrated using a solvent peak as internal reference. The following abbreviations are used to indicate the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Compound **3** was prepared as previously described.²³ Compound **10**, 1-adamantylacetic acid, was purchased from AK Scientific, Inc (Union City, CA).

3-(Adamantan-1-ylmethyl)-5,5-dimethyl-1,4,2-dioxazole (5)—Compound **1** (210 mg, 1 mmol) was added to the mixture of 2,2-dimethoxypropane (2 mL) and camphorsulfonic acid (116 mg, 0.5 mmol) in dry CH_2Cl_2 (5 mL) at rt and stirred for 10 h. Flash chromatography on silica (Hex/EtOAc = 10/1 $\rightarrow$ 5/1) afforded **5** (145 mg, 58% yield). ^1H NMR (500 MHz, CDCl_3) δ 1.58 (s, 6H, 2xMe); 1.60–1.72 (m, 12H, 6x CH_2 -Adm); 1.98 (m, 3H, 3xCH-Adm); 2.06 (s, 2H, CH_2CO). ^{13}C NMR (126 MHz, CDCl_3) δ 158.6, 114.4, 42.5, 37.9, 36.8, 32.9, 28.7, 25.2. ESI⁺-HRMS (m/z) calc. for $\text{C}_{15}\text{H}_{23}\text{NO}_2$ ($\text{M}+\text{H}$)⁺: 250.1801, found: 250.1803.

3-(Adamantan-1-ylmethyl)-5,5-bis(methyl- d_3)-1,4,2-dioxazole (5')—Compound **1** (210 mg, 1 mmol) was added to the mixture of acetone- d_6 (0.37 mL, 5 mmol), trimethyl orthoformate (0.55 mL, 5 mmol), camphorsulfonic acid (116 mg, 0.5 mmol) in dry CH_2Cl_2 (5 mL) at rt and stirred for 10 h. Flash chromatography on silica (Hex/EtOAc = 10/1 $\rightarrow$ 5/1) afforded product **5'** (130 mg, 51% yield). Deuterium content >95 % (according to the proton NMR). ^1H NMR (500 MHz, CDCl_3) δ 1.60–1.72 (m, 12H, 6x CH_2 -Adm); 1.98 (m, 3H, 3xCH-Adm); 2.06 (s, 2H, CH_2CO). ^{13}C NMR (151 MHz, CDCl_3) δ 158.6, 114.2, 42.5, 37.9, 36.8, 32.9, 28.7, 24.4 (septet). ESI⁺-HRMS (m/z) calc. for $\text{C}_{15}\text{H}_{17}\text{D}_6\text{NO}_2$ ($\text{M}+\text{H}$)⁺: 256.2172, found: 256.2180.

2-(Adamantan-1-yl)acetic-2,2- d_2 acid (10')—Commercially available adamantylacetic acid **10** (390 mg, 2 mmol) was added to the mixture of benzene- d_6 (4 mL) and 2 M AlCl_3 in nitrobenzene (1.1 mL). This mixture was microwaved at 125 °C for 30 min. Reaction mixture was diluted with CH_2Cl_2 and washed with water. Flash chromatography on silica (Hex/EtOAc = 5/1 $\rightarrow$ 1/1) afforded deuterated acid **10'** (295 mg, 75% yield). Deuterium content >95 % (according to the proton NMR). ^1H NMR (500 MHz, CDCl_3) δ 1.65–1.72 (m, 12H, 6x CH_2 -Adm); 1.98 (m, 3H, 3xCH-Adm). ^{13}C NMR (126 MHz, DMSO) δ 178.7,

48.3 (quintet), 42.4, 36.8, 32.7, 28.7. ESI⁺- HRMS (*m/z*) calc. for C₁₂H₁₆D₂O₂ (M+H)⁺: 197.1503, found: 197.1502.

2-(Adamantan-1-yl)-N-hydroxyacetamide-2,2-d₂ (3')—Deuterated acid **10'** (200 mg, 1 mmol) was dissolved in benzene (4 mL) and SOCl₂ (0.22 mL, 3 mmol) was added to this solution at rt. Next, 1 drop of DMF was added and the reaction mixture stirred at 80 °C for 1 h. Volatiles were evaporated and the resulting crude acyl chloride was re-dissolved in dry THF (5 mL). This solution was added drop wise to a well-stirred mixture of 50% *aq.* NH₂OH (1 mL, ~ 15 mmol) and THF (3 mL) at 0 °C. The reaction mixture was stirred overnight at rt. This mixture was then diluted with ethyl acetate (~ 50 mL) and washed with 1M (*aq.*) HCl (2 × 15 mL), water and brine. The organic phase was dried (MgSO₄) and volatiles were evaporated. Pure hydroxamic acid **3'** was obtained by re-crystallization from Et₂O/Hexanes (170 mg, 80% yield). Deuterium content >95 % (according to the proton NMR). ¹H NMR (600 MHz, CDCl₃) δ 1.64–1.76 (m, 12H, 6xCH₂-Adm); 1.96 (m, 3H, 3xCH-Adm). ¹³C NMR (151 MHz, DMSO) δ 170.6, 47.6 (quintet), 43.6, 37.9, 33.6, 30.2. ESI⁺-HRMS (*m/z*) calc. for C₁₂H₁₇D₂NO₂ (M+H)⁺: 212.1612, found: 212.1612.

Synthesis of carbamoyl-hydroxamates

Method A—Compound **3** (210 mg, 1 mmol) was dissolved in dry THF (4 mL) under inert atmosphere. Next, benzylisocyanate (0.14 mL, 1.1 mmol) was added at rt and stirred overnight. Volatiles were evaporated and the residue was chromatographed on a silica gel column (Hex/EtOAc = 4/1 → 1/2). Two regioisomers were obtained: less polar *N*-protected derivative **7** (173 mg, 50% yield) and *O*-protected prodrug **6a** (52 mg, 15% yield). Structures were confirmed by H/N-HSQC NMR.

Method B—Hydroxamic acid **3** or **3'** (1 mmol) was dissolved in dry MeCN (7 mL) under inert atmosphere. Next, 1,1'-carbonyldiimidazole (180 mg, 1.1 mmol) was added in one portion at 10–15 °C and stirred at this temperature for 20 min (TLC showed complete conversion to 1,4,2- dioxazol-5-one). Subsequent aminolysis (at 15–22 °C, 6–12 h) of the intermediate by addition of an amine (1.1 mmol) led to the formation of only *O*-protected prodrug. The reaction mixture was diluted with EtOAc (~50 mL) and washed with 0.5 M (*aq.*) HCl (2 × 10 mL), water, and brine. The organic phase was dried, volatiles were evaporated and the residue was chromatographed on silica gel column (Hex/EtOAc = 4/1 → 1/2).

2-(1-Adamantyl)-N-(benzylcarbamoyl)-N-hydroxyacetamide (7): Prepared by *Method A* (50% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.57–1.67 (m, 12H, 6xCH₂-Adm); 1.90 (m, 3H, 3xCH-Adm); 2.55 (s, 2H, CH₂CO); 4.33 (d, J = 6.1 Hz, 2H, CH₂-Bn); 7.23–7.32 (m, 5H, CH-Bn); 8.67 (t, J = 5.9 Hz, 1H, NH-Bn); 10.26 (s, 1H, N-OH). ¹³C NMR (151 MHz, CDCl₃) δ 173.3, 153.9, 137.5, 128.9, 127.8, 127.7, 47.4, 44.7, 42.5, 36.9, 34.1, 28.8. ESI⁺-HRMS (*m/z*) calc. for C₂₀H₂₆N₂O₃ (M+H)⁺: 343.2016, found: 343.2019. The structure was confirmed by H/N-HSQC NMR.

2-(1-Adamantyl)-N-[(benzylcarbamoyl)oxy]acetamide (6a): Prepared by *Method A* (15% yield) or *Method B* (88% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.58–1.67 (m, 12H, 6xCH₂-Adm); 1.82 (s, 2H, CH₂CO); 1.91 (m, 3H, 3xCH-Adm); 4.23 (d, J = 6.1 Hz, 2H, CH₂-Bn); 7.25–7.35 (m, 5H, CH-Bn); 8.18 (t, J = 6.1 Hz, 1H, NH-Bn); 11.25 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.4, 155.3, 139.1, 128.3, 127.0, 127.0, 46.3, 44.1, 41.9, 36.4, 32.2, 28.0. ESI⁺-HRMS (*m/z*) calc. for C₂₀H₂₆N₂O₃ (M+H)⁺: 343.2016, found: 343.2014. The structure was confirmed by H/N-HSQC NMR.

2-(1-Adamantyl)-N-[(benzylcarbamoyl)oxy]acetamide, [2,2-*d*₂] (6a'): Prepared by *Method B* (85% yield). Deuterium content > 95% (according to the proton NMR). ¹H NMR (600 MHz, CDCl₃) δ 1.62–1.70 (m, 12H, 6xCH₂-Adm); 1.97 (m, 3H, 3xCH-Adm); 4.40 (d, J = 5.9 Hz, 2H, CH₂-Bn); 5.82 (br-s, 1H, NH-Bn); 7.28–7.34 (m, 5H, CH-Bn); 8.91 (br-s, 1H, NH-O). ¹³C NMR (151 MHz, CDCl₃) δ 169.8, 155.3, 137.4, 128.9, 127.9, 127.7, 47.0 (quintet), 45.6, 42.5, 36.8, 28.7. ESI⁺-HRMS (*m/z*) calc. for C₂₀H₂₄D₂N₂O₃ (M+H)⁺: 345.2140, found: 345.2144.

2-(1-Adamantyl)-N-[(4-methoxybenzyl)carbamoyl]oxy]acetamide (6b): Prepared by *Method B* (88% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.57–1.66 (m, 12H, 6xCH₂-Adm); 1.81 (s, 2H, CH₂CO); 1.91 (m, 3H, 3xCH-Adm); 3.73 (s, 3H, CH₃O); 4.15 (d, J = 6.1 Hz, 2H, CH₂-Bn); 6.89 (d, J = 8.7 Hz, 2H, CH-Bn); 7.19 (d, J = 8.6 Hz, 2H, CH-Bn); 8.11 (t, J = 6.0 Hz, 1H, NH-Bn); 11.23 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.4, 158.3, 155.2, 131.1, 128.4, 113.7, 55.1, 46.3, 43.5, 41.9, 36.4, 32.2, 28.0. ESI⁺-HRMS (*m/z*) calc. for C₂₁H₂₈N₂O₄ (M+H)⁺: 373.2122, found: 373.2110.

2-(1-Adamantyl)-N-[(4-methoxybenzyl)carbamoyl]oxy]acetamide, [2,2-*d*₂] (6b'): Prepared by *Method B* (93% yield). Deuterium content ~90% (according to the proton NMR). ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.65 (m, 12H, 6xCH₂-Adm); 1.91 (s, 3H, 3xCH-Adm); 3.73 (s, 3H, CH₃O); 4.15 (d, J = 6.0 Hz, 2H, CH₂-Bn); 6.89 (d, J = 8.3 Hz, 2H, CH-Bn); 7.19 (d, J = 7.9 Hz, 2H, CH-Bn); 8.11 (t, J = 6.1 Hz, 1H, NH-Bn); 11.24 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.4, 158.3, 155.2, 131.1, 128.4, 113.7, 55.1, 45.7, 43.6, 41.9, 36.4, 32.1, 28.0; ESI⁺-HRMS (*m/z*) calc. for C₂₁H₂₇D₂N₂O₄(M+H)⁺: 375.2245, found: 375.2251.

2-(1-Adamantyl)-N-[(4-fluorobenzyl)carbamoyl]oxy]acetamide (6c): Prepared by *Method B* (54% yield). ¹H NMR (600 MHz, CDCl₃) δ 1.62–1.70 (m, 12H, 6xCH₂-Adm); 1.97 (m, 5H, CH₂CO and 3xCH-Adm); 4.36 (d, J = 4.2 Hz, 2H, CH₂-Bn); 5.95 (br-s, 1H, NH-Bn); 7.01 (m, 2H, CH-Bn); 7.25 (m, 2H, CH-Bn); 8.87 (br-s, 1H, NH-O). ¹³C NMR (151 MHz, CDCl₃) δ 169.9, 162.5 (d, *J*_{C-F} = 245.9 Hz), 155.3, 133.3 (d, *J*_{C-F} = 2.0 Hz), 129.4 (d, *J*_{C-F} = 8.2 Hz), 115.8 (d, *J*_{C-F} = 21.5 Hz), 47.6, 44.9, 42.5, 36.8, 33.1, 28.7. ESI⁺-HRMS (*m/z*) calc. for C₂₀H₂₅FN₂O₃ (M+H)⁺: 361.1922, found: 361.1925.

2-(1-Adamantyl)-N-[(4-fluorobenzyl)carbamoyl]oxy]acetamide, [2,2-*d*₂] (6c'): Prepared by *Method B* (77% yield). Deuterium content ~90% (according to the proton NMR). ¹H NMR (600 MHz, CDCl₃) δ 1.50–1.68 (m, 12H, 6xCH₂-Adm); 1.90 (s, 3H, 3xCH-Adm); 4.20 (d, J = 6.1 Hz, 2H, CH₂-Bn); 7.15 (t, J = 8.8 Hz, 2H); 7.30 (dd, J = 8.5, 5.6 Hz, 2H); 8.20 (t, J = 6.2 Hz, 1H); 11.26 (s, 1H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.4, 161.2 (d, *J*_{C-F} = 243 Hz), 155.3, 135.3, 129.1 (d, *J*_{C-F} = 7.55 Hz), 115.1 (d, *J*_{C-F} = 21.1 Hz), 45.7, 43.4, 41.9, 36.4, 32.1, 28.0. ESI⁺-HRMS (*m/z*) calc. for C₂₀H₂₄D₂FN₂O₃(M+H)⁺: 363.2045, found: 363.2039.

2-(1-Adamantyl)-N-[(ethylcarbamoyl)oxy]acetamide (6d): Prepared by *Method B* (87% yield). ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.03 (m, 3H, CH₃); 1.57–1.66 (m, 12H, 6xCH₂-Adm); 1.80 (s, 2H, CH₂CO); 1.91 (m, 3H, 3xCH-Adm); 3.04 (m, 2H, CH₂-NH); 7.60 (s, 1H, NH-Et); 11.17 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.3, 154.7, 46.3, 41.9, 36.4, 35.5, 32.2, 28.0, 14.9. ESI⁺-HRMS (*m/z*) calc. for C₁₅H₂₄N₂O₃ (M+H)⁺: 281.1860, found: 281.1869.

2-(1-Adamantyl)-N-[(ethylcarbamoyl)oxy]acetamide, [2,2-*d*₂] (6d'): Prepared by *Method B* (96% yield). Deuterium content ~90% (according to the proton NMR). ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.02 (m, 3H, CH₃); 1.49–1.69 (m, 12H, 6xCH₂-Adm); 1.90 (s, 3H,

3xCH-Adm); 3.03 (m, 2H, $\text{CH}_2\text{-NH}$); 7.59 (s, 1H, NH-Et); 11.16 (s, 1H, NH-O). ^{13}C NMR (151 MHz, $\text{DMSO-}d_6$) δ 167.3, 154.7, 45.7, 41.9, 36.4, 35.5, 32.1, 28.0, 14.9. ESI⁺-HRMS (m/z) calc. for $\text{C}_{15}\text{H}_{23}\text{D}_2\text{N}_2\text{O}_3(\text{M}+\text{H})^+$: 283.1983, found: 283.1981.

2-(1-Adamantyl)-N-[(*tert*-butylcarbamoyl)oxy]acetamide (6e): Prepared by *Method A* (32% yield). ^1H NMR (600 MHz, CDCl_3) δ 1.34 (s, 9H, ^tBu); 1.62–1.70 (m, 12H, 6x $\text{CH}_2\text{-Adm}$); 1.96 (s, 2H, CH_2CO); 1.97 (m, 3H, 3xCH-Adm); 5.28 (br-s, 1H, NH-Bn); 8.90 (br-s, 1H, NH-O). ^{13}C NMR (151 MHz, CDCl_3) δ 169.5, 153.1, 51.6, 47.7, 42.5, 36.8, 33.0, 28.8, 28.7. ESI⁺-HRMS (m/z) calc. for $\text{C}_{17}\text{H}_{28}\text{N}_2\text{O}_3(\text{M}+\text{H})^+$: 309.2173, found: 309.2179.

2-(1-Adamantyl)-N-[(*tert*-butylcarbamoyl)oxy]acetamide, [2,2- d_2] (6e’): Prepared by *Method B* (76% yield). Deuterium content > 95 % (according to the proton NMR). ^1H NMR (600 MHz, CDCl_3) δ 1.34 (s, 9H, ^tBu); 1.61–1.70 (m, 12H, 6x $\text{CH}_2\text{-Adm}$); 1.97 (m, 3H, 3xCHAdm); 5.42 (br-s, 1H, NH-Bn); 9.18 (br-s, 1H, NH-O). ^{13}C NMR (151 MHz, CDCl_3) δ 169.5, 153.2, 51.6, 47.1 (quintet), 42.5, 36.8, 32.9, 28.8, 28.7. ESI⁺-HRMS (m/z) calc. for $\text{C}_{17}\text{H}_{26}\text{D}_2\text{N}_2\text{O}_3(\text{M}+\text{H})^+$: 311.2296, found: 311.2299.

2-(1-Adamantyl)-N-[(morpholine-4-carbonyl)oxy]acetamide (6f): Prepared by *Method B* (82% yield). ^1H NMR (600 MHz, $\text{DMSO-}d_6$) δ 1.56–1.65 (m, 12H, 6x $\text{CH}_2\text{-Adm}$); 1.83 (s, 2H, CH_2CO); 1.91 (m, 3H, 3xCH-Adm); 3.38 (overlapped with water signal, $\text{CH}_2\text{-morpholine}$); 3.58 (m, 4H, 2x $\text{CH}_2\text{-morpholine}$); 11.37 (br-s, 1H, NH-O). ^{13}C NMR (151 MHz, $\text{DMSO-}d_6$) δ 167.5, 153.5, 65.8, 46.3, 44.2, 41.9, 36.4, 32.3, 28.0. ESI⁺-HRMS (m/z) calc. for $\text{C}_{17}\text{H}_{26}\text{N}_2\text{O}_4(\text{M}+\text{H})^+$: 323.1965, found: 323.1966.

2-(1-Adamantyl)-N-[(morpholine-4-carbonyl)oxy]acetamide, [2,2- d_2] (6f’): Prepared by *Method B* (85% yield). Deuterium content > 95% (according to the proton NMR). ^1H NMR (600 MHz, $\text{DMSO-}d_6$) δ 1.57–1.66 (m, 12H, 6x $\text{CH}_2\text{-Adm}$); 1.91 (m, 3H, 3xCH-Adm); 3.40 (m, 4H, 2x $\text{CH}_2\text{-morpholine}$); 3.58 (m, 4H, 2x $\text{CH}_2\text{-morpholine}$); 11.36 (br-s, 1H, NH-O). ^{13}C NMR (151 MHz, $\text{DMSO-}d_6$) δ 167.4, 153.5, 65.8, 45.6 (quintet), 44.2, 41.8, 36.4, 32.1, 28.0. ESI⁺-HRMS (m/z) calc. for $\text{C}_{17}\text{H}_{24}\text{D}_2\text{N}_2\text{O}_4(\text{M}+\text{H})^+$: 325.2089, found: 325.2088.

2-(1-Adamantyl)-N-[(dimethylcarbamoyl)oxy]acetamide (6g): Prepared by *Method B* (94% yield). ^1H NMR (500 MHz, CDCl_3) δ 1.62–1.70 (m, 12H, 6x $\text{CH}_2\text{-Adm}$); 1.97 (m, 5H, CH_2CO and 3xCH-Adm); 2.97 and 3.04 (2 x s, 2 x 3H, 2 x CH_3); 8.80 (br-s, 1H, NH-O). ^{13}C NMR (126 MHz, CDCl_3) δ 169.9, 155.4, 47.6, 42.5, 37.4, 36.8, 36.0, 33.0, 28.8. ESI⁺-HRMS (m/z) calc. for $\text{C}_{15}\text{H}_{24}\text{N}_2\text{O}_3(\text{M}+\text{H})^+$: 281.186, found: 281.1864.

2-(1-Adamantyl)-N-[(dimethylcarbamoyl)oxy]acetamide, [2,2- d_2] (6g’): Prepared by *Method B* (92% yield). Deuterium content ~ 95% (according to the proton NMR). ^1H NMR (600 MHz, CDCl_3) δ 1.63–1.70 (m, 12H, 6x $\text{CH}_2\text{-Adm}$); 1.98 (m, 3H, 3xCH-Adm); 2.97 and 3.04 (2 x s, 2 x 3H, 2 x CH_3); 8.70 (br-s, 1H, NH-O). ^{13}C NMR (151 MHz, CDCl_3) δ 169.9, 155.4, 47.0 (quintet), 42.5, 37.4, 36.8, 36.0, 32.9, 28.7. ESI⁺-HRMS (m/z) calc. for $\text{C}_{15}\text{H}_{22}\text{D}_2\text{N}_2\text{O}_3(\text{M}+\text{H})^+$: 283.1983, found: 283.1987.

2-(1-Adamantyl)-N-[(bis(2-methoxyethyl)carbamoyl)oxy]acetamide (6h): Prepared by *Method B* (77% yield). ^1H NMR (600 MHz, $\text{DMSO-}d_6$) δ 1.57–1.66 (m, 12H, 6x $\text{CH}_2\text{-Adm}$); 1.82 (s, 2H, CH_2CO); 1.91 (m, 3H, 3xCH-Adm); 3.24 and 3.25 (2 x s, 2 x 3H, 2 x CH_3O); 3.42 (m, 6H, 2x $\text{CH}_2\text{-O}$ and $\text{CH}_2\text{-N}$); 3.52 (m, 2H, $\text{CH}_2\text{-N}$); 11.36 (s, 1H, NH-O). ^{13}C NMR (151 MHz, $\text{DMSO-}d_6$) δ 167.5, 154.3, 70.2, 69.6, 58.2, 58.1, 48.0, 46.8, 46.2, 41.9, 36.4, 32.2, 28.0. ESI⁺-HRMS (m/z) calc. for $\text{C}_{19}\text{H}_{32}\text{N}_2\text{O}_5(\text{M}+\text{H})^+$: 369.2384, found: 369.2391.

2-(1-Adamantyl)-N-[(bis(2-methoxyethyl)carbamoyl)oxy]acetamide, [2,2-*d*₂] (6h')

Prepared by *Method B* (74% yield). Deuterium content ~ 95% (according to the proton NMR). ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.57–1.66 (m, 12H, 6xCH₂-Adm); 1.91 (m, 3H, 3xCH-Adm); 3.24 and 3.25 (2 x s, 2 x 3H, 2 x CH₃O); 3.42 (m, 6H, 2xCH₂-O and CH₂-N); 3.52 (m, 2H, CH₂-N); 11.35 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.5, 154.3, 70.2, 69.6, 58.2, 58.1, 48.0, 46.8, 45.6 (quintet), 41.9, 39.5, 36.4, 32.1, 28.0. ESI⁺-HRMS (*m/z*) calc. for C₁₉H₃₀D₂N₂O₅ (M+H)⁺: 371.2507, found: 371.2507.

2-(1-Adamantyl)-N-[(phenylcarbamoyl)oxy]acetamide (6i):

Prepared by *Method B* (58% yield). ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.55–1.70 (m, 12H, 6xCH₂-Adm); 1.84–1.98 (m, 5H, CH₂CO and 3xCH-Adm); 7.05 (t, *J* = 7.3 Hz, 1H, CH-*p*-Ph); 7.32 (t, *J* = 7.9 Hz, 2H, CH-*m*-Ph); 7.39–7.53 (m, 2H, CH-*o*-Ph); 10.18 (s, 1H, NH-Ph); 11.50 (s, 1H, NH-O). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 167.5, 152.2, 138.3, 128.9, 123.0, 118.3, 46.2, 41.9, 36.3, 32.3, 28.0; ESI⁺-HRMS (*m/z*) calc. for C₁₉H₂₅N₂O₃ (M+H)⁺: 329.1868, found: 329.1862.

2-(1-Adamantyl)-N-[(phenylcarbamoyl)oxy]acetamide, [2,2-*d*₂] (6i')

Prepared by *Method B* (66% yield). Deuterium content ~ 90% (according to the proton NMR). ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.57–1.67 (m, 12H, 6xCH₂-Adm); 1.93 (m, 3H, 3xCH-Adm); 7.04 (m, 1H, CH-*p*-Ph); 7.32 (m, 2H, CH-*m*-Ph); 7.45 (m, 2H, CH-*o*-Ph); 10.18 (s, 1H, NH-Ph); 11.50 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.6, 152.3, 138.3, 129.0, 123.1, 118.3, 45.5 (brquintet), 41.9, 36.4, 32.1, 28.0. ESI⁺-HRMS (*m/z*) calc. for C₂₁H₂₈N₂O₄ (M+H)⁺: 331.1983, found: 331.1984.

2-(1-Adamantyl)-N-[(4-methoxyphenyl)carbamoyl]oxy]acetamide (6j):

Prepared by *Method B* (39% yield). ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.49–1.73 (m, 12H, 6xCH₂-Adm); 1.79–2.07 (m, 5H, CH₂CO and 3xCH-Adm); 3.72 (s, 3H, MeO); 6.90 (d, *J* = 8.9 Hz, 2H, CH-*m*-Ph); 7.35 (d, *J* = 8.8 Hz, 2H, CH-*o*-Ph); 9.97 (s, 1H, NH-Ph); 11.44 (s, 1H, NH-O). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 167.5, 155.2, 152.4, 131.2, 119.9, 114.1, 55.2, 46.2, 41.9, 36.3, 32.2, 28.0; ESI⁺-HRMS (*m/z*) calc. for C₂₀H₂₇N₂O₄ (M+H)⁺: 359.1965, found: 359.1960.

2-(1-Adamantyl)-N-[(4-methoxyphenyl)carbamoyl]oxy]acetamide, [2,2-*d*₂] (6j')

Prepared by *Method B* (40% yield). Deuterium content ~90 % (according to the proton NMR). ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.57–1.67 (m, 12H, 6xCH₂-Adm); 1.92 (m, 3H, 3xCH-Adm); 3.72 (s, 3H, MeO); 6.90 (d, *J* = 8.7 Hz, 2H, CH-*m*-Ph); 7.35 (d, *J* = 8.7 Hz, 2H, CH-*o*-Ph); 9.97 (s, 1H, NH-Ph); 11.44 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.5, 155.2, 152.4, 131.3, 119.9, 114.2, 55.2, 45.7 (br-quintet), 41.9, 36.4, 32.1, 28.0. ESI⁺-HRMS (*m/z*) calc. for C₂₁H₂₈N₂O₄ (M+H)⁺: 361.2089, found: 361.2090.

2-(1-Adamantyl)-N-[(4-fluorophenyl)carbamoyl]oxy]acetamide (6k):

Prepared by *Method B* (56% yield). ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.52–1.70 (m, 12H, 6xCH₂-Adm); 1.81–1.96 (m, 5H, CH₂CO and 3xCH-Adm); 7.16 (t, *J* = 8.9 Hz, 2H, CH-*m*-Ph); 7.34–7.58 (m, 2H, CH-*o*-Ph); 10.22 (s, 1H, NH-Ph); 11.51 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.6, 158.0 (d, *J*_{C-F} = 240 Hz), 152.4, 134.7 (d, *J*_{C-F} = 3.02 Hz), 120.1, 115.6 (d, *J*_{C-F} = 22.7 Hz), 46.3, 41.9, 36.4, 32.3, 28.0; ESI⁺-HRMS (*m/z*) calc. for C₁₉H₂₄N₂O₃ (M+H)⁺: 347.1765, found: 347.1765.

2-(1-Adamantyl)-N-[(4-fluorophenyl)carbamoyl]oxy]acetamide, [2,2-*d*₂] (6k')

Prepared by *Method B* (61% yield). Deuterium content ~ 92% (according to the proton NMR). ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.54–1.70 (m, 12H, 6xCH₂-Adm); 1.88–1.96 (m, 3H, 3xCH-Adm); 7.16 (t, *J* = 8.9 Hz, 2H, CH-*m*-Ph); 7.33–7.56 (m, 2H, CH-*o*-Ph); 10.22 (s, 1H, NH-Ph); 11.50 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.6, 158.0 (d, *J*_{C-F}

= 242 Hz), 152.4, 134.7 (d, J_{C-F} = 15.1 Hz), 120.1, 115.6 (d, J_{C-F} = 30.2 Hz), 45.5, 41.9, 36.4, 32.1, 28.0; HRMS (ESI⁺) m/e calc'd for [M+H]⁺ C₁₉H₂₂D₂FN₂O₃: 349.1889, found 349.1884.

2-(1-Adamantyl)-N-[(3,5-dimethoxyphenyl)carbamoyl]oxy]acetamide (6l): Prepared by *Method B* (85% yield). ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.54–1.69 (m, 12H, 6xCH₂-Adm); 1.87 (s, 2H, CH₂CO), 1.92 (s, 3H, 3xCH-Adm); 3.70 (s, 6H, 2 x MeO); 6.22 (t, J = 2.2 Hz, 1H, CH-*p*-Ph); 6.67 (d, J = 2.2 Hz, 2H, CH-*o*-Ph); 10.13 (s, 1H, NH-Ph); 11.49 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.6, 160.7, 152.1, 140.0, 96.9, 94.8, 55.1, 46.2, 41.9, 36.4, 32.3, 28.0; ESI⁺-HRMS (m/z) calc. for C₂₁H₂₉N₂O₅ (M+H)⁺: 389.2071, found: 389.2077.

2-(1-Adamantyl)-N-[(3,5-dimethoxyphenyl)carbamoyl]oxy]acetamide, [2,2-*d*₂] (6l'): Prepared by *Method B* (78% yield). Deuterium content ~ 90% (according to the proton NMR). ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.60–1.67 (m, 12H, 6xCH₂-Adm); 1.93 (m, 3H, 3xCH-Adm); 3.71 (s, 6H, 2 x MeO); 6.22 (m, 1H, CH-*p*-Ph); 6.68 (m, 2H, CH-*o*-Ph); 10.13 (s, 1H, NH-Ph); 11.49 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.6, 160.7, 152.1, 140.0, 96.9, 94.8, 55.1, 45.7 (br-quintet), 41.9, 36.4, 32.1, 28.0. ESI⁺-HRMS (m/z) calc. for C₂₁H₂₈N₂O₄ (M+H)⁺: 391.2194, found: 391.2189.

2-(1-Adamantyl)-N-[(4-methylcoumarin-7-yl)carbamoyl]oxy]acetamide (6m): Diphosgene (0.12 mL, 1 mmol) was added to a suspension of 7-amino-4-methylcoumarin (175 mg, 1 mmol) in dry THF (10 ml) at rt and stirred for 15 min. Then the reaction mixture was heated to 50 °C and stirred for additional 45 min. The reaction mixture was cooled to 10 °C and compound **3** (210 mg, 1 mmol) was added together with ¹Pr₂EtN (0.38 mL, 2.2 mmol). The mixture was stirred at rt overnight and the reaction was quenched with water and the product extracted with EtOAc. The organic phase was dried, concentrated and column chromatography was accomplished on silica (Hex/EtOAc = 2/1 → 0/1) affording product **6m** (255 mg, 62% yield). The structure was confirmed by H/N-HSQC NMR. ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.57–1.68 (m, 12H, 6xCH₂-Adm); 1.89 (s, 2H, CH₂CO); 1.94 (m, 3H, 3xCH-Adm); 2.40 (d, J = 1.1 Hz, 3H, CH₃); 6.27 (d, J = 1.2 Hz, 1H, H-3'); 7.41 (dd, J = 8.7, 2.1 Hz, 1H, H-6'); 7.51 (d, J = 2.1 Hz, 1H, H-8'); 7.74 (d, J = 8.7 Hz, 1H, H-5'); 10.72 (s, 1H, NH-Bn); 11.65 (s, 1H, NH-O). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.7, 159.9, 153.8, 153.2, 152.2, 141.8, 126.3, 115.0, 114.4, 112.3, 104.8, 46.2, 41.9, 36.4, 32.3, 28.0, 18.0. ESI⁺-HRMS (m/z) calc. for C₂₃H₂₆N₂O₅ (M+H)⁺: 411.1914, found: 411.1914.

Stability Studies

Stock solutions of prodrugs and corresponding internal standards were prepared as 10 mM DMSO solutions. An appropriately diluted stock solution was added to PBS, pH 7.4 or neurobasal cell culture medium to make a 10–100 μM solution with a total DMSO concentration of 1%. **PBS Buffer:** At timed intervals, 50 μL aliquots were withdrawn and diluted with 46 μL of water. Appropriately diluted deuterated internal standard solution (4 μL) was added to this solution to serve as 1 μM standard. The solution was analyzed by LC/MS. **Culture medium:** At timed intervals, 50 μL aliquots were withdrawn and diluted with 195 μL of cold acetonitrile. Appropriately diluted deuterated internal standard solution (5 μL) was added to this solution to serve as 1 μM standard. The solution was vigorously mixed by vortex and its supernatant was taken for LC/MS analysis. **LC/MS:** Agilent 1100 LC/MS system equipped with Agilent ZORBAX C8 column was used for the analysis with the gradient of 5% to 99% B from 0 to 10 min at 0.5 mL/min, 99% to 100% from 10 to 10.1 min at 0.7 mL/min (A to B, where A = 0.1% formic acid in water, B = 0.1% formic acid in acetonitrile). The column was equilibrated for 3 min before the next sample injection. The MS acquisition included a 2 min delay. **Ionization of prodrugs and internal standards:** all

prodrugs and internal standards were analyzed by LC/MS in scan mode to determine the mass for single ion monitoring. In all cases, $[M+Na]^+$ peak was observed. **Analysis and Half-life Determination:** Each aliquot solution was analyzed by the LC/MS system using positive single ion monitoring (SIM) of prodrug+ Na^+ , internal standard+ Na^+ , 210.1 (non-deuterated hydroxamic acid), and 212.2 (deuterated hydroxamic acid). Observed area ratio was corrected by the correction factor (*vide infra*) to represent the percentage of prodrug and hydroxamic acid in the sample. Half-life was determined from a non-linear fit (exponential decay) of prodrug percentage versus time using GraFit software. **Calibration:** Several mixtures of each non-deuterated compound and its internal standard at different concentrations were prepared and analyzed by LC/MS in SIM mode. The correction factor was determined by the linear fit of concentration ratio versus observed area ratio.

Neuronal Cell-based Inhibition Assays

Pure BoNT/A was prepared from *C. botulinum* strains Hall A hyper as previously described.⁴⁸ The toxin was dissolved in PBS, pH 7.4 with 40 % glycerol, and stored at -20°C until use. Activity of the BoNT/A preparation was determined by the mouse bioassay^{49, 50} and specific toxicity was $\sim 1.25 \times 10^8$ mouse LD₅₀ Units/mg.

The hiPSC-derived neurons and culture medium were purchased from Cellular Dynamics International (Madison, WI), and cultured in 96-well plates as described for 5 days prior to the assay.²⁷ For inhibition assays, 200–250 LD₅₀ Units of BoNT/A1 was added to the cells in 50 μl stimulation medium (modified neurobasal containing 2.2 mM CaCl_2 and 56 mM KCl (Invitrogen) supplemented with B27 and glutamax), and the cells were incubated at 37°C in a humidified 5 % CO_2 atmosphere for 7.5–10 min. The cell culture medium containing toxin was removed, cells were washed 3 times with 200 μl of culture medium, inhibitors were added in culture medium with a final DMSO concentration of 1%, and the cells were incubated for 8 h at 37°C , 5 % CO_2 . Next, culture medium was removed and cells were lysed in 50 μl of 1 X LDS buffer (Invitrogen). Samples were analyzed by Western blot analysis using a monoclonal anti- SNAP-25 antibody (Synaptic Systems, Germany) as described previously,^{51, 52} except PhosphaGLO (KPL, Inc.) was used as the chemiluminescent substrate and bands were visualized on a FOTO/Analyst FX (FOTODYNE, Inc.) equipped with a CCD camera and GraphQuant software for densitometric analysis. Data analysis was performed using GraphPad Prism 6 software.

Cytotoxicity

The cytotoxicity of compounds on neuronal cells was determined by Western blot analysis as described *vide supra*. Compounds were incubated with cells in the presence and/or absence of BoNT and the amount of SNAP-25 was quantified. A significant loss or absence of the SNAP-25 protein signal was determined to be caused by cell death.

Confocal Laser Scanning Microscopy

Neuro-2a cells (ATCC CCL-131) were cultured in Eagle's minimum essential medium (2 mM L-glutamine) supplemented with 10% fetal bovine serum at 37°C in a humidified, 5% CO_2 environment. For microscopy studies, Neuro-2a cells were grown on circular 12 mm coverslips coated with fibronectin ($\sim 1.5 \times 10^5$ cells). After overnight incubation, cell culture medium was removed and replaced with fresh medium containing 50 μM compound **6m** or free AMC in a final DMSO concentration of 1% (control cells - DMSO only), and the cells were incubated for 3 h. Following treatment, cells were fixed for 30 min in 4% formaldehyde and permeabilized in 0.2% Triton X-100 for 5 min. After blocking for 30 min in 2% goat serum-PBS, cells were labeled with Alexa Fluor 488-conjugated wheat germ agglutinin followed by TOTO-3 (Molecular Probes) and stored in Prolong Gold antifade

reagent (Invitrogen) until analysis on a Zeiss LSM 710 laser scanning confocal microscope (LSCM) attached to a Zeiss Observer Z1 microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BoNT/A	botulinum neurotoxin serotype A
LC/A	botulinum neurotoxin light chain serotype A
SNAP-25	synaptosomal-associated protein of 25 kDa
hiPSC	human induced pluripotent stem cells
SAHA	suberoylanilide hydroxamic acid
AMC	7-amino-4-methylcoumarin
CDI	1,1'-carbonyldiimidazole
SIL	stable isotopically labeled
LD₅₀	Lethal Dose 50%
PBS	phosphate buffered saline

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Figure 1. Hydroxamic acid inhibitors of LC/A displaying nanomolar inhibition constants: **1**, **2**, **3**, **4**.^{23–26}

Figure 2.
Structures of known anti-viral (amantadine and rimantadine) and anti-cancer (SAHA) drugs.

Figure 3. Inhibition of BoNT/A induced SNAP-25 cleavage in hiPSC-derived neurons: negative control (–control) - cells without BoNT/A; positive control (+control) - cells plus BoNT/A; prodrugs **5** and **6a** - cells plus BoNT/A followed by 25 μ M compound.

Figure 4. Inhibition of BoNT/A induced SNAP-25 cleavage in hiPSC-derived neurons. (A) Prodrug **6a** shows dose-dependent inhibition with an EC₅₀ value of 20 μ M. (B) The presence of 0.01 % Tween-80 does not affect the EC₅₀ value or inhibition profile of prodrug **6a**.

Figure 5.

Internalization of prodrug **6m** and subsequent release of AMC (blue) in Neuro-2a cells as viewed by laser scanning confocal microscopy. (A1) Neuro-2a cells were incubated with 50 μ M **6m**, fixed, and labeled with Alexa Fluor 488-conjugated wheat germ agglutinin (green) and TOTO-3 (far-red). (A2) Cross section Z-slice from A1. (B) Neuro-2a cells alone were treated in the same manner as in A1.

Scheme 1. Synthesis of 1,4,2-Dioxazole and Carbamate Prodrugs.^a

^a(a) $\text{CH}_3\text{C}(\text{OMe})_2\text{CH}_3$, camphorsulfonic acid (0.5 equiv.), CH_2Cl_2 , rt, 10 h; (b) Bn-NCO (1.1 equiv.), THF, rt, overnight; ratio **6a**:**7** = 1:3.5.

Scheme 2. Synthesis of Carbamoyl hydroxamates **6a-l via Intermediate **8**.^a**

^a(a) CDI (1.1 equiv.), MeCN, 10–15 °C, 25 min; (b) amine (1.1 equiv.), rt, 6–12 h.

Scheme 3. Synthesis of Fluorogenic Prodrug 6m.^a

^a(a) (i) diphosgene (1.1 equiv.), THF, rt → 50 °C, 1 h; (ii) **3** (1 equiv.), ⁱPr₂NEt (2.2 equiv.).

Scheme 4. Synthesis of Deuterium-labeled Analogs.^a

^a(a) Acetone-d₆, (MeO)₃CH, camphorsulfonic acid (0.5 equiv.), CH₂Cl₂, rt, 10 h; (b) 0.5 M solution in benzene-d₆, AlCl₃ (1.1 equiv. in Ph-NO₂), microwave, 125°C, 30 min; (c) (i) SOCl₂ (3 equiv.), DMF (1 drop), benzene, 80° C, 1 h; (ii) NH₂OH (15 equiv., 50% sol. in H₂O), 0°C -> rt, 8 h.

Table 1

Stability, Log P, and Cellular Activity of Prodrugs.

Entry	Compound	$t_{1/2}(\text{PBS})^a$ [h]	$t_{1/2}(\text{medium})^b$ [h]	Log P^c	EC ₅₀ [μM]
1	5	3.3 $\pm$ 0.5	10.2 $\pm$ 0.9	3.99	>100
2	6a	17.6 $\pm$ 0.6	14.6 $\pm$ 0.9	3.52	20
3	6b	14 $\pm$ 3	11.2 $\pm$ 0.4	3.55	42
4	6c	11.5 $\pm$ 1.6	11.5 $\pm$ 0.7	3.64	27
5	6d	27.6 $\pm$ 1.5	15.1 $\pm$ 1.0	2.53	>100
6	6e	72 $\pm$ 8	N.D.	3.53	N.D. (toxic)
7	6f	48 $\pm$ 1	33 $\pm$ 5	2.22	37
8	6g	149 $\pm$ 9	61 $\pm$ 3	2.38	>100
9	6h	164 $\pm$ 5	48 $\pm$ 3	2.39	>100
10	6i	1.1 $\pm$ 0.1	0.76 $\pm$ 0.04	3.80	>100
11	6j	1.1 $\pm$ 0.2	0.85 $\pm$ 0.06	3.83	>100
12	6k	0.9 $\pm$ 0.1	0.62 $\pm$ 0.05	3.92	>100
13	6l	1.2 $\pm$ 0.1	1.6 $\pm$ 0.1	3.83	>100

N.D. = not determined;

^a half-life in PBS;

^b half-life in culture medium;

^c average of calculated Log P^{46}