Published in final edited form as:

Bioorg Med Chem. 2011 November 1; 19(21): 6203–6209. doi:10.1016/j.bmc.2011.09.019.

Formulating a new basis for the treatment against botulinum neurotoxin intoxication: 3,4-diaminopyridine prodrug design and characterization

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Abstract

Botulism is a disease characterized by neuromuscular paralysis and is produced from botulinum neurotoxins (BoNTs) found within the Gram positive bacterium *Clostridium botulinum*. This bacteria produces the most deadliest toxin known, with lethal doses as low as 1 ng/kg. Due to the relative ease of production and transport, the use of these agents as potential bioterrorist weapons has become of utmost concern. No small molecule therapies against BoNT intoxication have been approved to date. However, 3,4-diaminopyridine, (3,4-DAP), a potent reversible inhibitor of voltage-gated potassium channels, is an effective cholinergic agonist used in the treatment of neuromuscular degenerative disorders that require cholinergic enhancement. 3,4-DAP has also been shown to facilitate recovery of neuromuscular action potential post botulinum intoxication by blocking K⁺ channels. Unfortunately, 3,4-DAP displays toxicity largely due to blood-brain-barrier (BBB) penetration. As a dual-action prodrug approach to cholinergic enhancement we have designed carbamate and amide conjugates of 3,4-DAP. The carbamate prodrug is intended to be a slowly reversible inhibitor of acetylcholinesterase (AChE) along the lines of the stigmines thereby allowing increased persistence of released acetylcholine within the synaptic cleft. As a secondary activity, cleavage of the carbamate prodrug by AChE will afford the localized release of 3,4-DAP, which in turn, will enhance the pre-synaptic release of additional acetylcholine. Being a competitive inhibitor with respect to acetylcholine, the activity of the prodrug will be greatest at the synaptic junctions most depleted of acetylcholine. Here we report upon the synthesis and biochemical characterization of three new classes of prodrugs intended to limit previously reported stability and toxicity issues. Of the prodrugs examined, compound **32**, demonstrated the most clinically relevant half-life of 2.76 h, while selectively inhibiting AChE over butyrylcholinesterase

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at doi:.

– a plasma-based high activity esterase. Future *in vivo* studies could provide validation of prodrug **32** as a potential treatment against BoNT intoxication as well as other neuromuscular disorders.

Keywords

Botulinum neurotoxins; Acetylcholinesterase inhibitors; 3,4-DAP; prodrug

1. Introduction

Botulinum neurotoxins (BoNTs) produced from the Gram positive bacterium *Clostridium botulinum* are some of the deadliest toxins known to man¹ with lethal doses as low as 1 ng per kg body weight being 10 million times more toxic than cyanide.² Intoxication by BoNT produces generalized muscle weakness, and in severe cases flaccid paralysis leading to impaired respiration and autonomic function. In many cases rapid intubation and mechanical respiration are required to prevent greater risk and even death.³ BoNT intoxication is primarily caused by the consumption of poisoned canned food products; however, the use of these agents as potential bioterrorist weapons has become of concern. As such, the US Centers for Disease Control (CDC) have classified BoNTs as one of the six highest-risk agents for bioterrorism (Class A).

The induction of neuromuscular paralysis by BoNTs requires three biochemical steps. First, BoNT protein binds to gangliosides on the presynaptic cholinergic nerve terminal through interactions with the heavy chain. These interactions allow subsequent endocytosis into the neuron through several possible mechanisms involving synaptotagmins I and II (BoNT/B and BoNT/G) and SV2 (BoNT/A).⁴ The toxin is then translocated into the cytosol where its light chain (LC), a metalloprotease, binds to, and cleaves soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor proteins (SNAREs).⁵ This action halts the release of acetylcholine (ACh) at the neuromuscular junction, leading to the cessation of neurotransmission.

Currently, the only approved therapies against BoNT intoxication include pre-exposure prophylaxis with a vaccine and post-exposure administration of sera containing anti-BoNT antibodies.⁶ Upon cellular intoxication, however, it is imperative to provide fast acting neuro-modulatory drugs to recover neurotransmission through ACh release, to at least restore partial muscle function. Thus, a potential small molecule pharmacological treatment could provide many benefits over these antibody-based approaches. Most small molecule research efforts have targeted the metallo-proteolytic properties of the BoNT LC protease, however, no small molecule therapeutics have been approved to date.⁷ Yet, clinically approved cholinergic enhancing drugs have been implemented to treat similar neurological disorders including Lambert-Eaton myasthenic syndrome (LEMS),⁸ multiple sclerosis (MS),⁹ and Alzheimer's disease.¹⁰

Of these classes of drugs, aminopyridines, in particular 3,4-diaminopyridine (3,4-DAP), has shown promise due to its agonistic effect on neurotransmitter release through K_v channel blockade.¹¹ The aminopyridines, 3,4-DAP along with 4-aminopyridine (4-AP) facilitate recovery of neuromuscular action potential post botulism intoxication by reversibly blocking voltage-dependent K⁺ channels.¹² This action promotes Ca²⁺ influx, driving signal transduction and ACh release at the synapse. The mechanism by which aminopyridines inactivate the K_v channel is unknown; however, using molecular modeling Caballero and colleagues hypothesized that two putative receptor sites found within the tetrameric channel are important in this overall process.¹³ It has also been hypothesized that once

aminopyridines cross the membrane, the molecules become protonated intracellularly, thereby allowing the molecule to interact with receptors within the pore of the channel.

Recently, our laboratory initiated studies toward deciphering the pK_a /hydrogen-bonding properties of 3,4-DAP in conjunction with the K_v channel.¹⁴ By synthesizing structural analogues, we hoped to discover more potent, yet, less toxic forms of the pharmacophore for treatment against BoNT intoxication. Previous reports indicate that 3,4-DAP and 4-AP, when administered at high doses, demonstrate toxicity issues such as seizures due to the molecules' ability to penetrate the blood-brain barrier (BBB). As such, the development of new compounds that alleviate BBB penetration without hampering the necessary molecular dynamics between the compound and the K_v channel would provide a path forward for treating BoNT/A intoxication. Exploring this possibility, a series of compounds were synthesized, and 3,4,5-triaminopyridine and 3,4-diamino-1-(prop-2-ynyl)pyridinium were found to exhibit therapeutic potential, as characterized by paralysis reversal in mouse phrenic nerve-hemidiaphragm assays post BoNT/A induced paralysis. Yet, a shortcoming with these molecules is that they possessed modest plasma half-lives of 0.31 h and 1.2 h, respectively. Drug candidates with limited plasma lifetimes would require repeated dose regimen or a continuous iv infusion to systemically provide paralytic extinction.

While many research programs have focused on inhibition of the BoNT LC protease itself, therapies directed toward the inhibition of acetylcholinesterase (AChE) post BoNT intoxication remain sparse.¹⁵

The stigmines, a class of carbamate-based AChE inhibitors, provide effective treatment of multiple sclerosis,¹⁶ myasthenia gravis,¹⁷ and Alzheimer's disease.¹⁸ Mechanistically, carbamates produce reversible covalent inhibition via transesterification between the carbamoyl-moiety and the active site serine. Hydrolysis of the serine-carbamate intermediate is considerably slower (seconds to tens of minutes) than that of the acetyl-serine adduct formed during substrate hydrolysis. Clinically approved stigmines such as Rivastigmine (Exelon) are characterized as pseudo-substrate inhibitors of both AChE and serum-based butyrylcholinesterase (BChE), a highly active esterase with largely overlapping substrate specificity that must be avoided for therapeutic efficacy.¹⁹ Although obtaining selectivity for AChE over BChE is a challenging task, the success of the stigmines suggests that prodrug-type molecules could be similarly designed with sufficient selectivity towards AChE over BChE.

From the structure activity relationship found within the stigmines, carbamate conjugates of 3,4-DAP could act as potential pseudo-substrate inhibitors of AChE thereby providing the enhanced benefit of selectively unmasking 3,4-DAP proximal to its site of action. Also of note, carbamate inhibitors are competitive with respect to ACh meaning that inhibition of AChE and liberation of 3,4-DAP will occur in those synaptic junctions most depleted (and in need) of ACh. AChE is homeostatically occupied by ACh ($K_m = 0.4$ mM) within the neuromuscular junction.²⁰ Interestingly, studies by Smart and McCammon have found that the deactivation of AChE increases ACh lifetime in the synaptic cleft from 200 to 900 μ s.²¹ Acetyl choline depletion could produce up to a 25-fold inhibition selectivity for AChE at BoNT/A intoxicated neuromuscular junctions over undamaged junctions. Therefore, there could be a therapeutically relevant directed inhibition of AChE and 3,4-DAP liberation in those synaptic clefts most depleted of ACh by BoNT intoxication.

Herein, we describe our studies regarding the design, synthesis and *in vitro* study of 3,4-DAP-based dual-acting prodrugs against BoNT intoxication.

1. Materials and Methods

2.1 Materials

Acetylthiocholine iodide (AChI), S-Butyrylthiocholine iodide (BChI), 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), and sera from clotted mouse blood were purchased from Sigma Aldrich (St. Louis, MO). 100 mM stocks of AChI, BChI, and DTNB were prepared in 100 mM sodium phosphate pH 8.0. All other chemicals used were of analytical grade and highest chromatographic purity available. AChE purified from *Electrophorus electricus* (Electric eel) – Type V-S and BChE from equine serum were purchased from Sigma Aldrich and reconstituted in 1% gelatin/100 mM sodium phosphate pH 8.0 at 500 U/mL each.

2.2 Enzyme inhibition assays

The activities of class I, II, and III inhibitors were measured according to the method of Ellman et al.²² using AChI or BChI as the respective substrates for each enzyme. In brief, 100 μ M AChI or BChI was added to 100 mM sodium phosphate pH 8.0 containing 360 μ M DTNB and a range of prodrug concentrations from 500 μ M to 2 μ M. To initiate the reaction, 0.5 U of enzyme was added to each sample in a final volume of 200 μ l per well in Costar 3904 96 well plates (Corning Inc., Corning, NY). Enzyme rates were measured at 37°C over the linear range using a Spectromax 250 spectrophotometer with 412 nm absorbance readings. (All reagent stocks were prepared in 100 mM sodium phosphate pH 8.0 unless stated otherwise).

In order to determine the kinetic mechanism of inhibition, inhibitors and substrate(s) were run in varying concentrations bracketing their K_i s and K_m s respectively.

2.3 Enzyme-specific drug release assays

To determine enzyme-mediated drug release, 500 μ M of prodrug was incubated in 100 mM sodium phosphate pH 8.0 containing 1.0 U enzyme (100 μ L total volume) for 3 h at 37°C. Post incubation, samples were quenched with 17.4 M glacial acetic acid and analyzed by analytical reverse-phase HPLC using a Vydac C₁₈ 218TP54 column (Western Analytical Products, Lake Elsinore, CA) monitored at 254 and 320 nm. A linear gradient of 0.5–50% (v/v) of solvent B (0.09% v/v TFA/acetonitrile) in solvent A (0.1% v/v TFA/water) from 5 to 15 min, then 50–90% (v/v) B over 5 min was used for all compounds. Peak area and linear dynamic range used to calculate 3,4-DAP was by use of authentic standards. Prodrugs demonstrating greater 3,4-DAP release by AChE over BChE were further analyzed for half-life in mouse sera.

2.4 Sera half-life assays

15 μ l of a 10 mM prodrug stock was added to 85 μ L sera, pre-warmed to 37°C. Samples were then incubated at the following fixed time intervals 0, 5, 10, 20, 40, 60, and 120 min. Upon extraction with 300 μ L 0 °C acetonitrile, precipitated proteins were removed by centrifugation (20,817 rcf) and the liquid phase was subsequently analyzed by analytical reverse-phase HPLC as described above. Stability of prodrug was determined based on presence of breakdown product 3,4-DAP. Half-life values were calculated using the 1-hour endpoint samples.

2.5 Statistical analysis

The prodrug concentration producing the 50% AChE and BChE activity inhibition (IC₅₀), along with inhibition constant K_i values were calculated by non-linear regression analysis using GraphPad Prism v.5.0b software (GraphPad Software Inc., CA).

3. Results and Discussion

3.1 Design of Prodrugs

Based on previous reports of SAR and plasma stability, three prodrug models were designed with the hope of discovering dual action AChE inhibitors providing the selective release of 3,4-DAP at the site of intoxication. Class I molecules were designed from substrate specificity studies and modeled such that the anionic binding site of AChE would be occupied by 3,4-DAP while the active site would be occupied by an ester (Figure 1). Upon ester catalyzed hydrolysis, the prodrug would degrade to release 3,4-DAP into the synaptic cleft to act on nearby K_v channels (Figure 1). Class II prodrugs, on the other hand, were designed to contain a carbamoyl scissile linker like the stigmines. Lastly, class III prodrugs were designed to test whether non-specific esterases/peptidases in sera would allow the release of 3,4-DAP slowly over time.

3.2 Synthesis of Class I prodrugs

The synthesis of Class I prodrugs followed one of two paths. Class Ia prodrugs (**6a-i**) began from 4-hydroxybenzaldehyde (**1**), which was treated with various alkyl acid chlorides affording compounds **2a-i**, (Scheme 1). Subsequent reduction with NaBH_4 resulted in benzyl alcohols **3a-i**. Reaction of aryl azide **4**,²³ and subsequently its Curtius rearrangement; the resulting isocyanate was trapped by alcohols **3a-i** to provide carbamates **5a-i**. Finally, reduction of the nitro group with stannous chloride afforded DAP prodrugs **6a-i**. Here yields ranged from 4% to 32% over four steps.

The general synthesis of Class Ib prodrugs followed a similar sequence. For compound **10**, 1,2-phenylenedimethanol, (**7**), was treated with isobutyryl chloride affording **8**, (Scheme 2^a). Next, alcohol **8** underwent reaction with **4** providing carbamate **9**. The nitro moiety was subsequently reduced yielding **10** in an overall yield of 9% over three steps.

On the other hand, the synthesis of carbamate **14** began from ethylene glycol (**11**), which was converted to ester **12** using isobutyryl chloride. Conversion to nitrocarbamate **13**, vide supra, followed by its reduction, (Scheme 2^b), afforded **14** in 12% yield over three steps.

3.3 Synthesis of Class II prodrugs

The construction of the class II prodrugs began with the reaction of commercially available 3,4-DAP (**15**) and methyl chloroformate to provide carbamates **16** and **17** in one pot, (Scheme 3), which upon separation resulted in yields of 10% and 22% respectively. The 3,4-DAP prodrug **19**, in which the carbamate is located at the 4-position, was prepared via palladium-catalyzed hydrogenation of the known precursor **18**,²⁴ with a yield of 36%.

3.4 Synthesis of Class III prodrugs

The synthesis of class III prodrugs are depicted in Scheme 4 and Scheme 5. Amide **21** was obtained via palladium-catalyzed hydrogenation of the known aminopyridine **20**,²⁵ (Scheme 4), 68% yield. Diamide **22** was prepared in one step from 3,4-DAP (**15**) using acetyl chloride.

The synthesis of amides **25** and **27** proceeded from a common, commercially available starting material, 3-nitro-4-aminopyridine (**23**). In pursuit of **25**, amide bond formation with benzoyl chloride yielded **24**, followed by reduction of the nitro group to provide **25**, with a yield of 18% over two steps. Synthesis of **27** began with the amide union between **23** and cyclopentanecarbonyl chloride, followed by reduction to provide **27** with a yield of 48% over two steps.

The final Class III prodrug, **32**, started from commercially available succinic anhydride (**28**), which was treated with isopropyl alcohol to form the ester **29**, followed by conversion to acyl chloride **30** (Scheme 5). The reaction of **30** with **23** in the presence of base provided **31**, which in turn was reduced to the desired primary amine **32** via palladium-catalyzed hydrogenation. The overall yield over this four-step synthesis was 17%.

3.5 Inhibition, release profile, and stability of prodrugs

From screens of candidate compounds against AChE and BChE, only compounds **6b** and **6d**, of the substrate-like class I prodrugs, demonstrated moderate selectivity of AChE over BChE, as well as AChE-dependent release of 3,4-DAP (Tables 1 and 2). Both compounds bound to AChE 100-fold more tightly than K_m for ACh. Progress curves of substrate consumption in the presence of the inhibitors were essentially linear indicating no significant buildup of acyl-enzyme intermediates (which would give time-dependent inhibition). While inhibition was relatively potent, turnover and release of 3,4-DAP was modest (Table 2). Incubation studies with AChE and BChE at saturating levels of **6b** and **6d** gave the expected zero order kinetics with apparent k_{cats} that were approximately 10^{-4} slower than the natural substrates ACh and BCh. Given the higher concentration of 3,4-DAP release by AChE, these two prodrugs were further tested for their respective half-lives in mouse sera. Unfortunately compounds **6b** and **6d** were unstable to incubation in sera, with half-lives of just 1.2 and 1.9 min respectively. The extremely short half-lives of these molecules are due to unidentified esterases of the labile esters found within the prodrug molecules. The structural similarity of all class I substrate-like prodrugs meant that no other compounds from this class were likely to be stable in mouse sera.

The carbamates most representative of our intended dual action strategy, compounds **16**, **17**, and **19** from class II prodrugs all produced moderate to poor inhibition profiles (IC_{50s} : 36–660 μ M) but failed as pseudosubstrates to generate 3,4-DAP enzymatically (Supporting Information Table S2). Simple methyl carbamates of 3,4-DAP are not recognized by the cholinergic esterases as stigmime-like substrates. As such, these carbamates were not analyzed for half-lives in sera.

Amide **21** from the class III prodrugs demonstrated competitive inhibition of AChE (32 μ M K_i). Not surprisingly, the less labile amide bond did not undergo internal release upon incubation with AChE for 3 hours. Amide **32**, while potently inhibiting AChE (3 μ M K_i) also demonstrated negligible release of 3,4-DAP over the 3 h incubation period (Table 2). The greater stability of the amide-conjugated 3,4-DAP gave this prodrug an appreciable half-life of 2.8 h in mouse sera, providing a path forward for *in vivo* analysis, as well as elucidating the core functionality necessary in the design and synthesis of additional 3,4-DAP prodrugs.

4. Conclusion

The design and synthesis of small molecule inhibitors of BoNT have been of great interest over the past decade.²⁶ While most compounds have focused on the direct inhibition of the BoNT protease, few have been designed to symptomatically relieve neuromuscular paralysis by blocking voltage-dependent potassium channels. This report took an alternative approach by investigating the possibility of dual action toward cholinergic enhancement at BoNT damaged synapses. We attempted to mask the K_v channel blocker 3,4-DAP as a carbamate or amide conjugated pseudo-substrate inhibitor of AChE thus allowing its directed delivery to afflicted synapses. The combined effect of the prodrug is to enhance the action potential for the neuron pre-synaptically as well as increase the lifetime of ACh post-synaptically. Although simple methylcarbamate conjugates to 3,4-DAP were not recognized by AChE as pseudo-substrates, the carbamoyl-linked 4-benzylalcohol-phenol esters **6b** and **6d** were.

These compounds bound relatively tightly to AChE (low μM) but were sluggish in their turnover by AChE having only 10^{-4} the k_{cat} of ACh. The esters within these compounds rendered them unstable to mouse sera.

Alternatively, amide conjugation of 3,4-DAP exemplified by **32**, resulted in compounds that inhibited AChE selectively over BChE with good potency but did not liberate 3,4-DAP through the intended internal release mechanism. The greater stability of the amide bond gave **32** a reasonable half-life (2.8 hours) for the release of 3,4-DAP in mouse sera. Although such compounds lack the dual action originally intended, masking 3,4-DAP for controlled release holds therapeutic promise. On its own, 3,4-DAP generates muscle action potential and muscle contraction *in vivo*, yet its ability to cross the BBB and produce seizures has questioned the potential therapeutic relevance of this molecule.²⁷ As such, the design of prodrugs of 3,4-DAP that limit toxicity would be pertinent as a means to effectively treat neuro-paralytic disorders including BoNT induced paralysis.

Three classes of prodrugs were designed, synthesized, and evaluated *in vitro* with the goal of elucidating structures that act as pseudosubstrates for AChE and release 3,4-DAP at affected neurons, via enzyme mediated catalysis. Localized 3,4-DAP would act to increase vesicular ACh release at intoxicated junctions, allowing the recovery of neuromuscular transmission while reduced acetylcholinesterase activity via AChE inhibition will allow the limited ACh present to persist. We anticipate that continual 3,4-DAP in circulation, released slowly overtime, would nullify the previous inefficacies of concentrated, repeated dosing, as well as BBB penetration.

Of the 19 compounds synthesized, two demonstrated inhibition and selectivity between enzyme classes, as well as a drug-release profile necessary for half-life determination in mouse sera (**6b** and **6d**). Unfortunately, further testing revealed that these two class I prodrugs were unstable in sera limiting their usefulness *in vivo*. Prodrug **32** on the other hand, demonstrated inhibition and selectivity for AChE, but did not act as a pseudosubstrate for the enzyme. Regardless, **32** was tested for half-life in sera to determine if unidentified esterases/peptidases present could degrade the prodrug while in circulation, ultimately releasing 3,4-DAP over time. Prodrug **32** in fact had the best half-life of 2.76 h. The stability of **32** in sera may possibly be due to its amide bond, which would not be readily cleaved within the active site of AChE, but may be hydrolyzed by non-specific peptidases in sera. Modeling studies may help to clarify the chemical properties necessary for binding and release via AChE. In sum, **32** could be used as a lead structure to elucidate more potent inhibitors. However, future *in vivo* experiments will be necessary to validate prodrug **32** as an effective treatment for BoNT intoxication and other neuromuscular disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by a postdoctoral fellowship from the German Academic Exchange Service (DAAD) to D.G., as well as The Skaggs Institute for Chemical Biology, and federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under award number AI080671 (K.D.J.).

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Figure 1.

Top^a. Proposed mechanism of class I 3,4-DAP prodrugs. *Bottom^b*. Representative carbamate and amide class II and III inhibitors and AChE active site mechanism of action.

Scheme 1.

Synthesis of Class Ia prodrugs.

Conditions: (a) RCOCl , Et_3N , THF, $0\text{ }^\circ\text{C}$ to rt, 3 h; (b) NaBH_4 , THF, $0\text{ }^\circ\text{C}$ to rt, 0.5 – 2.5 h;(c) benzene, reflux, 0.5 – 1 h; (d) $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, MeCN, reflux, 1 h.

Scheme 2. Synthesis of Class Ib prodrugs **10** (Top) and **14** (Bottom).^aConditions: (a) isobutyryl chloride, Et₃N, CH₂Cl₂, rt, 16 h; (b) **4**, benzene, reflux, 0.5 h; (c) SnCl₂•2H₂O, MeCN, reflux, 1.5 h.^bConditions: (a) isobutyryl chloride, Et₃N, CH₂Cl₂, rt, 1 h; (b) benzene, reflux, 20 min; (c) H₂ (50 psi), Pd/C, EtOAc, rt, 14 h.

Scheme 3.

Synthesis of Class II prodrugs.

Conditions: (a) ClCO_2Me , DMA, rt, 5 h, 10; (b) H_2 (50 psi), Pd/C, EtOAc, rt, 48 h.

Scheme 4.

Synthesis of Class III prodrugs **21**, **22**, **25**, and **27**.

Conditions: (a) H_2 (60 psi), Pd/C, MeOH, rt, 16 h; (b) AcCl, DMA, rt, 14 h; (c) BzCl, Et₃N, CH₂Cl₂, 0 °C to rt, 14 h; (d) H_2 (50 psi), Pd/C, EtOAc, rt, 60 h; (e) cyclopentanecarbonyl chloride, Et₃N, CH₂Cl₂, 0 °C to rt, 60 h; (f) H_2 (50 psi), Pd/C, EtOAc, rt, 13 h.

Scheme 5. Synthesis of Class III prodrug **32**.

Conditions: (a) iPrOH, reflux, 14 h, then 150 °C, 3 h; (b) (COCl)₂, CH₂Cl₂, DMF, rt, 0.5 h; (c) **23**, Et₃N, CH₂Cl₂, rt, 15 h; (d) H₂ (50 psi), Pd/C, EtOAc, rt, 3 h.

Table 1*In vitro* inhibition profiles of **6b**, **6d**, and **32**.

Compd.	K_i^a	
	AChE	BChE
6b	5.7 ± 0.7	25 ± 3
6d	3.9 ± 0.6	42 ± 5
32	3.2 ± 0.6	62 ± 11

^a Calculated K_i values in μM . All data are represented as average $\pm$ S.E.M.

Table 2*In vitro* kinetic profiles of **6b**, **6d**, and **32**.

Compd.	Drug Release ^a		Sera Half-life ^b
	AChE	BChE	
6b	145	82	1.2
6d	105	82	1.9
32	3.4	5.8	165

^aConcentration of 3,4-DAP in μM post 3 h enzymatic hydrolysis.^bCalculated half-life values in min.