Synthesis and evaluation of library of betulin derivatives against the botulinum neurotoxin A protease

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ABSTRACT

Botulinum neurotoxins (BoNTs) are the most toxic proteins currently known. Current treatments for botulinum poisoning are all protein based with a limited window of opportunity. Inhibition of the BoNT light chain protease (LC) has emerged as a new therapeutic strategy for the treatment of botulism as it may provide an effective post-exposure remedy. As such, a small library of 40 betulin derivatives was synthesized and screened against the light chain of BoNT serotype A (LC/A); five positive hits (IC$_{50}$ <100 μM) were uncovered. Detailed evaluation of inhibition mechanism of three most active compounds revealed a competitive model, with sub-micromolar K_i value for the best inhibitor (7). Unfortunately, an in vitro cell-based assay did not show any protection of rat cerebellar neurons against BoNT/A intoxication by 7.

Botulinum neurotoxins (BoNTs), proteins produced by bacteria of the genus Clostridium,[1] are responsible for botulism, a disease characterized by peripheral neuromuscular blockade and a characteristic flaccid paralysis of humans. BoNTs are the most poisonous substances known, with serotype A having a lethal dose for a 70 kg human of approximately 0.09–0.15 μg intravenously or intramuscularly, and 0.7–0.9 μg inhalationally.[2] Despite their potentially lethal toxicity, BoNTs have emerged as an extremely valuable therapeutic tool for the treatment of a variety of maladies, including strabismus, migraines, and even facial wrinkles.[3] However, the potential use of BoNT in a bioterrorist attack remains imminent and the Center for Disease Control (CDC) now classifies this agent as 'category A', placing it among the six highest-priority agents. Current treatments for botulinum poisoning are all antibody based with a limited window of therapeutic effectiveness. A particular drawback with these vaccine approaches is that they cannot reverse the effects after the toxin has reached its target inside the cell.[4]

Inhibition of the BoNT light chain metalloprotease (LC) has surfaced as a new therapeutic strategy for the treatment of botulism as it may provide an effective post-exposure remedy. BoNTs are synthesized as ~150 kDa proteins that are post-translationally activated by proteolytic cleavage to form mature di-chain proteins consisting of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) linked by a disulfide bond.[5] The HC is responsible for the neurospecific binding, uptake, and translocation of the LC into the cytosol of neuronal cells. The LC is a Zn^{2+}-dependent metalloprotease that cleaves one of three intracellular soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins: syntaxin, vesicle-associated membrane protein (VAMP)/synaptobrevin, or synaptosomal-associated protein of 25 kDa (SNAP-25) depending on the serotype. As a consequence of protein cleavage, release of acetylcholine at the neuromuscular junction is blunted resulting in the loss of neurotransmission. Small molecule inhibitors of the LC may provide an opportunity for development of both pre- and post-exposure therapeutics. In recent years, the LC of serotype A (LC/A) has been a major focus, primarily due to its potency and long duration of paralysis.[4] A number of competitive inhibitors of LC/A have been reported the most potent have K_i values of 0.6–10 μM,[6] several of which coordinate the active site zinc cation required for catalysis (K_i ranging from 0.3 to 12.3 μM).[7] More recently, the natural product D-chicoric acid was discovered in our laboratory that putatively binds to an exosite region outside the LC/A active site, displaying noncompetitive partial inhibition.[8]
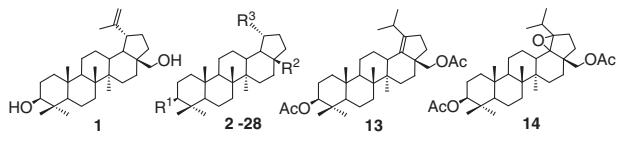
Betulin is a naturally occurring, pentacyclic triterpene alcohol belonging to the lupane series of compounds. Betulin is the

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Table 1
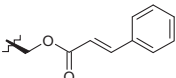Structures of betulin derivatives screened against BoNT/A protease
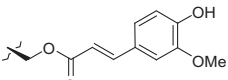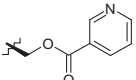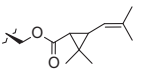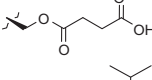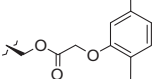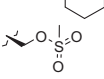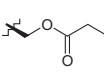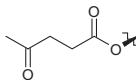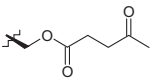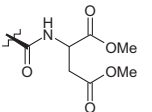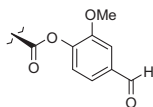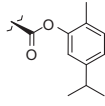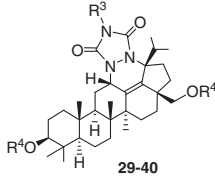
Compound	R^1	R^2	R^3
1	OH	CH$_2$OH	
2	OH	CH$_2$OH	
3	OH	(cinnamoyloxy methyl)	
4	OH	(4-hydroxy-3-methoxycinnamoyloxy methyl)	
5	OH	(nicotinoyloxy methyl)	
6	OH	(chrysanthemoyloxy methyl)	
7	OH	(succinyloxy methyl, CO$_2$H)	
8	OH	(aryloxyacetyloxy methyl)	
9	OH	OAc	
10	OAc	OH	
11	OAc	CH$_2$OAc	
12	OAc	CH$_2$OAc	(epoxide)
13[a]	OAc	CH$_2$OAc	
14[a]	OAc	CH$_2$OAc	
15	OAc	(tetrahydropyranyloxy methyl)	
16	OAc	(sulfonyl ester methyl)	
17	(levulinoyloxy)	(levulinoyloxy methyl)	
18	OH	CO$_2$H	
19	OH	CO$_2$Me	
20	O=	CHO	
21	O=	CO$_2$H	
22	O=	CO$_2$Me	
23	O=	CO$_2$H	
24	O=	(N-acyl aspartate diMe)	

Table 1 (continued)

Compound	R^1	R^2	R^3
25	O=	(3-OMe-4-CHO benzoyloxy methyl)	
26	O=	(2-methyl-5-isopropyl phenoxy methyl)	
27	OH	CH=NOH	
28	=NOH	CH=NOH	

Compound	R^3	R^4
29	Me	Ac
30	Me	CocHex
31	Me	COPh
32	n-Bu	Ac
33	Ph	Ac
34	Bn	Ac
35	3-MeO-Ph	Ac
36	4-F-Ph	Ac
37	3-NO$_2$-Ph	Ac
38	4-Ac-Ph	Ac
39	4-Cl-Ph	Ac
40	Indan-5-yl	Ac

[a] See structure above.

principle extractive substance of outer birch bark, and it has been extracted from white-barked birches (*Betula* sp.) in amounts up to 30% dry weight. Betulin **1** can be converted to betulinic acid **18**,[9] which has plethora of pharmacological properties, such as cytotoxic activity against several tumor cell lines by inducing apoptosis in cells.[10] Excitingly, some betulin derivatives have also shown remarkable anti-HIV activity with new mechanisms of action.[11] We have previously reported several new bioactivities for these highly interesting compounds, such as anti-leishmanial,[12] antialphaviral[13] and anti-chlamydial activity.[14] Finally, structure–activity relationship (SAR) studies and pharmacological properties of betulin and its derivatives have been recently reviewed.[15]

Based on previous studies we were intrigued about potential activity of such compounds against BoNT/A. Therefore, a library of 40 betulin derivatives was tested for their inhibition of BoNT/A protease. The chemical structures of the betulin-derived triterpenoids are presented in Table 1; we note that we have previously reported these compounds,[12a,13] with the exception of **4**, **7**, **12** and **26**, which are now provided in Supplementary data. Thus, compounds were tested at a single concentration (50 μM) by LC/MS assay[7b] at 10 μM concentration of optimized truncated SNAP-substrate (66-mer; 141–206 aa) encompassing the key recognition elements of SNAP-25. From this lot, five positive hits **7**, **8**, **18**, **19** and **21** (IC$_{50}$ <100 μM) were uncovered and three of these, **7**, **8** and **18** were further evaluated at various concentrations of substrate and inhibitor. Obtained data were most consistent with a competitive inhibition model (Supplementary data). The inhibition constants, K_i were determined by a non-linear least squares global fit to the initial rates of product formation for matrixes of substrate and inhibitor concentrations bracketing K_m and K_i,

Table 2
IC$_{50}$ and K_i values of positive hits

Compound	IC$_{50}$ (μM)	K_i (μM)
7	1.5	0.8 ± 0.2
8	10	13.3 ± 2.3
18	20	14.3 ± 2.2

N.D. = not determined.

respectively (Table 2). These results revealed that 28-hemisuccinylbetulin **7** was the best inhibitor with K_i = 0.8 ± 0.2 μM. Thus, making it interesting lead structure for iterative rounds of structural modification in search of more potent small molecule antagonists of BoNT/A. The other betulin derivatives, betulinyl 28-carboxymethoxycarvacrolate **8** and betulinic acid **18** (Table 1) were about 16 and 18-fold less potent, respectively.

Potency of the best inhibitor was further investigated using an in vitro cell-based assay that monitors intracellular cleavage of SNAP-25. Thus, the cellular efficacy of compound **7** was tested using primary rat cerebellar neurons at 30 and 80 μM concentrations, respectively. However, this assay did not show inhibitory activity of this derivative against BoNT/A. Clearly, cells are complex biological systems and other factors such as permeability can influence the efficacy of small molecules within cellular models. Additionally, we highlight that betulin derivatives possess very low solubility in aqueous buffers that can also influence cellular activity (see Supplementary data for calculated physicochemical data).

In summary, a small library of 40 betulin derivatives was prepared and subsequently tested for potential inhibition of BoNT/A protease. Interestingly, five compounds within this library were discovered to exert low micromolar activities against the protease. Further detailed evaluation of the mechanism of inhibition of the three most active compounds revealed competitive inhibition, with a sub-micromolar K_i value for the best inhibitor. Disappointingly, in vitro cell-based examination did not demonstrate protection of rat cerebellar neurons against BoNT/A intoxication by **7**. It is anticipated that additional modifications of the betulin structure to endow better water solubility and cell permeability will allow the discovery of betulins with cellular activity against BoNT/A.

Acknowledgments

Supplementary data

Supplementary data (experimental procedures, full characterization for compounds **4**, **7**, **12**, **26** and biological assay conditions) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.115.

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