Published in final edited form as:
Bioorg Med Chem Lett. 2011 April 15; 21(8): 2229–2231. doi:10.1016/j.bmcl.2011.02.115.

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 postexposure 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**.

Keywords

Betulin derivatives; Botulinum neurotoxin; Protease inhibitor

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

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Supplementary Material
Experimental procedures, full characterization for compounds **4**, **7**, **12**, **26** and biological assay conditions are available on-line.

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 – 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 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 tumour 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] anti-alphaviral[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 supporting information. 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 (Supp. Information). 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, respectively (Table 2). These results revealed that 28-hemisuccinylbetulin **7** was the best inhibitor with $K_i = 0.8 \pm 0.2$ 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 Supp. Information 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Mark S. Hixon and Adjunct Professor Salme Koskimies for very helpful discussions. This project was supported through the Skaggs Institute for Chemical Biology as well as with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract number N01-AI-30050 (C.B.S.) and award number AI082190 (K.D.J). The financial support from the Finnish Funding Agency for Technology and Innovation, Foundation for Research of Natural Resources in Finland, Marjatta ja Eino Kollin Säätiö, and the project by the European Commission (Contract No EU-KBBE-227239) are gratefully acknowledged (J.Y.-K.).

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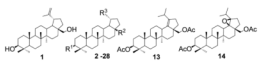
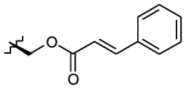
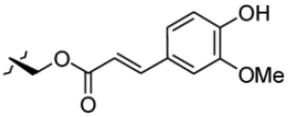
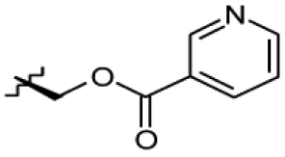
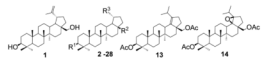
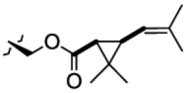
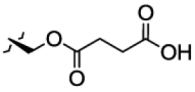
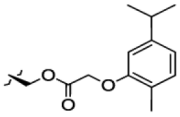
Table 1

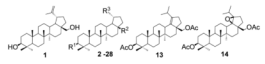
Structures of betulin derivatives screened against BoNT/A protease

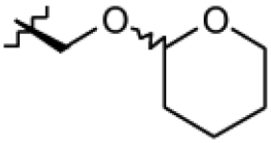
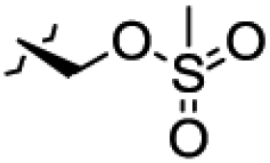
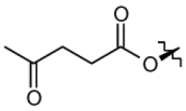
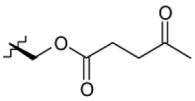


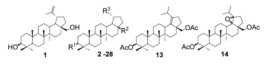
Compound	R^1	R^2	R^3
1	OH	CH_2OH	
2	OH	CH_2OH	
3	OH		
4	OH		
5	OH		

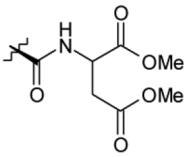


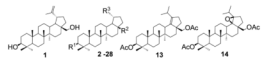
Compound	R^1	R^2	R^3
6	OH		
7	OH		
8	OH		
9	OH	OAc	
10	OAc	OH	
11	OAc	CH_2OAc	
12	OAc	CH_2OAc	

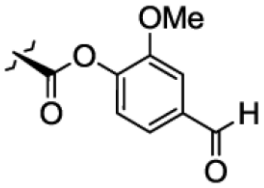
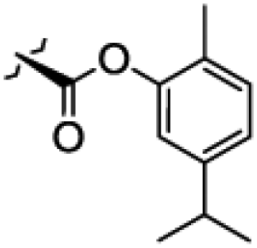


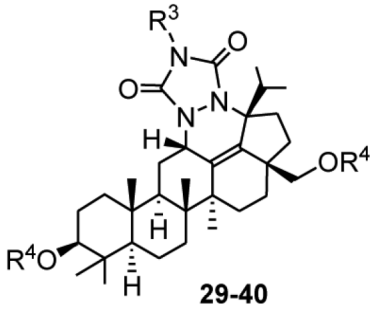
Compound	R^1	R^2	R^3
13 [2]	OAc	CH_2OAc	
14 [2]	OAc	CH_2OAc	
15	OAc		
16	OAc		
17			
18	OH	CO_2H	



Compound	R^1	R^2	R^3
19	OH	CO_2Me	
20	O=	CHO	
21	O=	CO_2H	
22	O=	CO_2Me	
23	O=	CO_2H	
24	O=		

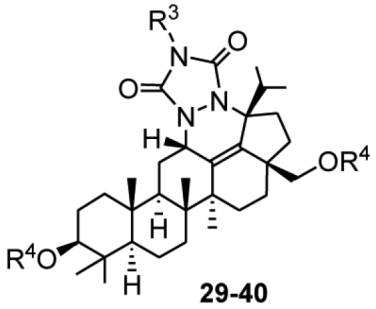


Compound	R^1	R^2	R^3
25	O=		
26	O=		
27	OH	CH=NOH	
28	=NOH	CH=NOH	



29-40

Compound	R^3	R^4
29	Me	Ac
30	Me	CocHex
31	Me	COPh
32	*n*-Bu	Ac
33	Ph	Ac



29-40

Compound	R^3	R^4
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

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