Published in final edited form as:

Int J Pharm. 2017 October 15; 531(2): 714–717. doi:10.1016/j.ijpharm.2017.07.070.

Inhibition of *Clostridium perfringens* epsilon toxin by β -cyclodextrin derivatives

Tanisha M. Robinson^{a,1}, Laszlo Jicsinszky^{a,b}, Andrei V. Karginov^c, and Vladimir A. Karginov^{a,2,*}

^aInnovative Biologics, Inc., Herndon, Virginia, USA

^bDipartimento di Scienza e Tecnologia del Farmaco, University of Turin, Turin, Italy

^cDepartment of Pharmacology, University of Illinois at Chicago, Illinois, USA

Abstract

Clostridium perfringens epsilon toxin (ETX) is considered as one of the most dangerous potential biological weapons. The goal of this work was to identify inhibitors of ETX using a novel approach for the inactivation of pore-forming toxins. The approach is based on the blocking of the target pore with molecules having the same symmetry as the pore itself. About 200 various β -cyclodextrin derivatives were screened for inhibitors of ETX activity using a colorimetric cell viability assay. Several compounds with dose-dependent activities at low micromolar concentrations have been identified. The same compounds were also able to inhibit lethal toxin of *Bacillus anthracis*.

Graphical abstract

Keywords

β -cyclodextrin derivatives; toxin inhibitors; *Clostridium perfringens*; ϵ -toxin; *Bacillus anthracis*; lethal toxin

*Corresponding author at: KFBio, LLC, 43136 Fling Ct., Ashburn, VA 20148. Tel.: +1 703 622 5749. vak@kfbllc.com (Vladimir A. Karginov).

¹Current address: Naval Medical Research Center, 503 Robert Grant Ave., Silver Spring, MD 20910.

²Current address: KFBio, LLC, 43136 Fling Ct., Ashburn, VA 20148.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Cyclodextrins are widely used as pharmaceutical agents to enhance the solubility, bioavailability, and stability of drug compounds because they can encapsulate organic molecules (Arima, 2017). Earlier, a new way of using cyclodextrin derivatives was suggested by us – utilizing them as direct antimicrobial agents (Karginov, 2005).

Many pathogens utilize the formation of pores in the membranes of target cells in the process of infection. It has been demonstrated that a great number of pore-forming proteins, both bacterial and viral, are important virulence factors and are broadly considered as valid targets for the discovery of new drugs (Los, 2013).

A novel approach to inhibit pore-forming toxins was developed by us, which is based on the blocking of the target pore with molecules having the same symmetry as the pore and comparable dimensions. It was successfully tested on various bacterial toxins forming heptameric trans-membrane pores, such as anthrax toxin, *Staphylococcus aureus* α -toxin, as well as other toxins produced by *Clostridium difficile* and *Clostridium botulinum*. β -cyclodextrin (β -CD) derivatives having seven-fold symmetry were used as pore blockers (Karginov, 2013).

The goal of this work was to identify the inhibitors of epsilon toxin (ETX) of *Clostridium perfringens* (*C. perfringens*) which is one of the most lethal bacterial toxins (Anderson, 2012). It is considered as a potential biological weapon and is included in the list of category B priority agents by the USA Centers for Disease Control and Prevention. Besides its biodefense importance, ETX-producing *C. perfringens* cause enterotoxemia in sheep, goats and other animals (Freedman, 2016; Popoff, 2011; Smedley *et al.*, 2004; Songer, 1996). Inhibitors of ETX can be used for the development of drugs for the treatment of natural *C. perfringens* infections in animals. Currently, no specific therapy exists for ETX (Stiles, 2013); therefore, a great need exists for the development of effective drugs against this toxin.

Since ETX forms heptameric pores in sensitive cells (Miyata, 2002) similarly to other toxins utilized as targets previously, it seemed logical to utilize the described above approach for the identification of inhibitors of this toxin. In the present study, about 200 various β -cyclodextrin derivatives were tested for the ability to protect sensitive cells from ETX action using a colorimetric cell viability assay.

2. Materials and methods

2.1. General description of the synthesis of the cyclodextrin derivatives

The scheme of the synthesis is presented in Figure 1. Reagents were purchased from Sigma-Aldrich. A standard ultrasonic water bath was used to accelerate the dissolutions.

Heptakis(6-deoxy-6-iodo)- β -cyclodextrin was prepared with the following modification of the known method (Gadelle and Defaye 1991) on a 25 mmol scale: The reaction mixture in dimethylformamide (DMF) was concentrated to about half of its volume at 60 °C/5–10 mbar and methanol (~20 fold of DMF content) was added to it at a temperature of 40–45 °C. The pH of the homogeneous red solution was adjusted to range of 9–10 by adding in small

portions of solid sodium methoxide using an ultrasonic water bath. As the pH became neutral, the product started to precipitate. The suspension was allowed to crystallize overnight. The product was filtered off and washed with methanol, then it was dried at 40 °C in the presence of KOH and phosphorous pentoxide. The dried crude was suspended in water and stirred for 30 minutes at room temperature, filtered and the solid was washed with water until the aqueous phase became neutral. The crystals were dried as above. The obtained light yellow product (yield: 83 %) showed traces of triphenyl phosphine related impurities and some percent of DMF in the ¹H-NMR spectrum and it was used without further purification.

Heptakis(6-N-alkylamino-6-deoxy)-β-cyclodextrins were prepared as is summarized in Figure 1 by the following method: The dried heptakis(6-deoxy-6-iodo)-β-cyclodextrin (0.38 g, 0.0002 mol) was added in small portions to the sonicated alkylamine (2 ml) at 50–55 °C. The next portion was added when the cyclodextrin halogenide was completely dissolved. As the addition was completed, the reaction mixture was immersed into an oil bath and stirred for 4 hours at 75–80 °C using a reflux cooler. The alkylamine was removed under reduced pressure and the red-brown oily residue was cooled to 0–5 °C then dissolved in 3 ml 1 N HCl. The pH of the solution was < 1 and clarified with charcoal (0.1 g) for 30 minutes at room temperature. Charcoal was removed by filtration and 2 ml of aqueous ammonia solution was added. The ammonia was allowed to evaporate overnight and the formed solid was filtered, washed with water and dried as above resulting in white to pale yellow solids (yields: 58–98 %) as summarized in Table 1.

Infrared spectroscopy and mass spectrometry analysis of the synthesized compounds are presented in supplementary data.

2.2. Cytotoxicity neutralization assays

Madin-Darby canine kidney cells (MDCK.2, ATCC No. CRL-2936) were cultured in T-75 vented-cap flasks (Corning, Inc., Lowell, MA) using Eagle Minimal Essential Medium (ATCC, Manassas, VA) containing 10% FBS (ATCC, Manassas, VA), per ATCC protocols.

Protection of the cells from the cytotoxic effect of ETX by β-cyclodextrin derivatives was monitored using a colorimetric cell viability assay that measures the conversion of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] in the presence of PMS (phenazine methosulfate) into a formazan product. MDCK.2 cells were seeded at 2×10⁴ cells/well in 96-well cell culture plates (Becton Dickinson, Franklin Lakes, NJ) and incubated overnight at 37°C with 5% CO₂. Appropriate concentrations of β-CD compounds were added to the wells, immediately followed by 4.7 ng of trypsin-activated ETX (provided by Dr. Bruce McClane, University of Pittsburg), and incubated for approximately 4 hours. Media was removed, cells washed twice using Dulbecco's Minimal Essential Medium (MediaTech, Manassas, VA), and then MTS/PMS solution (Promega Corporation, Madison, WI) was added. Plates were incubated overnight at room temperature and A₅₉₀ readings taken using an ELx808 Absorbance Microplate Reader (BioTek Instruments, Winooski, VT).

Protection of the murine macrophage RAW 264.7 cells from anthrax lethal toxin (LeTx) induced cell death was monitored as described in Karginov *et al.*, 2006.

3. Results and discussion

First, ~200 various β -cyclodextrin derivatives were screened for compounds that protected MDCK.2 cells from ETX action using the MTS bioreduction cell viability assay (Figure 2). A group of structurally related β -CD derivatives that inhibited ETX's cytotoxicity at low micromolar concentrations was identified. The compounds protected the cells in a dose-dependent fashion and exhibited relatively low cell toxicity. The same compounds were tested for the ability to protect cells against lethal toxin of *B. anthracis* and they showed excellent inhibiting activity (Table 2), although compound NM001 displayed some toxicity toward RAW 264.7 cells at higher concentrations (Figure 3).

Only a couple of articles on inhibitors of ETX have been published to date. In the first one (Pelish and McClain, 2009), mutants of ETX were utilized as dominant-negative inhibitors. However, this approach utilizes high molecular weight proteins. Such inhibitors are less attractive as potential drugs in comparison with low molecular weight compounds, which are more desirable given their better bioavailability, lack of sensitivity to proteases and lower cost of production. In another article (Lewis *et al.*, 2010), several small-molecule inhibitors of ETX were identified in a 151,616-compound chemical library. Unfortunately, the IC₅₀ values for the identified inhibitors were not determined due to limited solubility of the compounds in the tissue culture medium, so they could not be compared with the inhibitors found in this study. The lead compounds presented in current work could be used for the development of new drugs for treating intoxication by epsilon toxin. Also, it should be pointed out that the yield of active compounds from the relatively small number of β -cyclodextrin derivatives was extraordinary high. Interestingly, the same compounds also inhibited *B. anthracis* lethal toxin, and they can potentially be used for the development of a strategic drug targeting multiple types of pore-forming toxins.

Compound NM-026 [No. NR-33153 (NP β CD)] as well as two other β -CD based antimicrobials NR-33151 (ANBO β CD) and NR-33152 (AMBnT β CD) (not reported in this article) are available for research purposes through BEI Resources at ATCC. Please refer to the BEI Resources website for more details (www.beiresources.org).

4. Conclusions

As a result of this work, a reliable and robust cell-based assay was developed and utilized for the screening of about 200 various β -cyclodextrin derivatives and the identification of inhibitors of ETX. The obtained results showed that β -cyclodextrin derivatives can inhibit the cytotoxicity of ETX and may be further developed into new therapeutics against this and other pore-forming toxins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grant R44AI074105-02 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. The authors wish to thank Dr. Bruce McClane for providing *C. perfringens* epsilon toxin.

References

- Anderson PD. Bioterrorism: toxins as weapons. *J Pharm Pract.* 2012; 25(2):121–9. [PubMed: 22523138]
- Arima H, Motoyama K, Higashi T. Potential use of cyclodextrins as drug carriers and active pharmaceutical ingredients. *Chem Pharm Bull (Tokyo).* 2017; 65(4):341–348. [PubMed: 28381674]
- Freedman JC, McClane BA, Uzal FA. New insights into *Clostridium perfringens* epsilon toxin activation and action on the brain during enterotoxemia. *Anaerobe.* 2016; 41:27–31. [PubMed: 27321761]
- Gadelle A, Defaye J. Selective Halogenation at Primary Positions of Cyclomaltooligosaccharides and a Synthesis of Per-3,6-anhydro-Cyclomaltooligo-saccharides. *Angew Chem Int Ed Eng.* 1991; 30(1): 78–80.
- Karginov VA, Nestorovich EM, Moayeri M, Leppla SH, Bezrukov SM. Blocking anthrax lethal toxin at the protective antigen channel by using structure-inspired drug design. *Proc Natl Acad Sci USA.* 2005; 102:15075–15080. [PubMed: 16214885]
- Karginov VA, Nestorovich EM, Yohannes A, Robinson TM, Fahmi NE, Schmidtman F, Hecht SM, Bezrukov SM. Search for Cyclodextrin-Based Inhibitors of Anthrax Toxins: Synthesis, Structural Features, and Relative Activities. *Antimicrob Agents Chemother.* 2006; 50:3740–3753. [PubMed: 16982795]
- Karginov VA. Cyclodextrin derivatives as anti-infectives. *Curr Opin Pharmacol.* 2013; 13(5):717–25. [PubMed: 24011515]
- Lewis M, Weaver CD, McClain MS. Identification of Small Molecule Inhibitors of *Clostridium perfringens* ϵ -Toxin Cytotoxicity Using a Cell-Based High-Throughput Screen. *Toxins (Basel).* 2010; 2(7):1825–1847. [PubMed: 20721308]
- Los FC, Randis TM, Aroian RV, Ratner AJ. Role of pore-forming toxins in bacterial infectious diseases. *Microbiol Mol Biol Rev.* 2013; 77(2):173–207. [PubMed: 23699254]
- Miyata S, Minami J, Tamai E, Matsushita O, Shimamoto S, Okabe A. *Clostridium perfringens* epsilon-toxin forms a heptameric pore within the detergent-insoluble microdomains of Madin-Darby canine kidney cells and rat synaptosomes. *J Biol Chem.* 2002; 277(42):39463–8. [PubMed: 12177068]
- Popoff MR. Epsilon toxin: a fascinating pore-forming toxin. *FEBS J.* 2011; 278(23):4602–15. [PubMed: 21535407]
- Pelish TM, McClain MS. Dominant-negative inhibitors of the *Clostridium perfringens* epsilon-toxin. *J Biol Chem.* 2009; 284(43):29446–53. [PubMed: 19720828]
- Smedley JG 3rd, Fisher DJ, Sayeed S, Chakrabarti G, McClane BA. The enteric toxins of *Clostridium perfringens*. *Rev Physiol Biochem Pharmacol.* 2004; 152:183–204. [PubMed: 15517462]
- Songer JG. Clostridial enteric diseases of domestic animals. *Clin Microbiol Rev.* 1996; 9:216–234. [PubMed: 8964036]
- Stiles BG, Barth G, Barth H, Popoff MR. *Clostridium perfringens* epsilon toxin: a malevolent molecule for animals and man? *Toxins (Basel).* 2013; 5(11):2138–60. [PubMed: 24284826]

Figure 1.
Synthesis of per-6-deoxy-6-N(alkyl)amino- β -cyclodextrins.

Figure 2.

Protection of MDCK.2 cells from ETX-induced cytotoxicity by compound NM001. MDCK.2 cells were incubated with different concentrations of the compound with or without ETX. Cell viability was determined by MTS colorimetric cell proliferation assay.

Figure 3. Protection of Raw 264.7 cells from LeTx-induced cytotoxicity by compound NM001. Raw 264.7 cells were incubated with different concentrations of the compound with or without LeTx. Cell viability was determined by MTS colorimetric cell proliferation assay.

Table 1

Summary of synthesis and solubility of the prepared compounds.

Comp. name	Appearance	Yield g (%)	Solubility in media μM
NM001	white powder	0.28 (93)	> 200
NM026	light yellow powder	0.26 (80)	> 200
NM002	light yellow powder	0.20 (58)	> 200
NM006	light yellow powder	0.24 (68)	> 200

Table 2

Activities of cyclodextrin derivatives in this study.

Comp. name	R	Inhibition of ETX cytotoxicity IC ₅₀ (μM)	Inhibition of LeTx cytotoxicity IC ₅₀ (μM)
NM001		2.6±0.2	1.6±0.5
NM026		2.0±0.3	4.5±0.3
NM002		38±1	0.8±0.4
NM006		11±1	1.1±0.1