International Journal of Pharmaceutics 531 (2017) 714–717

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

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

Tanisha M. Robinson[a,1], Laszlo Jicsinszky[a,b], Andrei V. Karginov[c], Vladimir A. Karginov[a,*,2]

[a] Innovative Biologics, Inc., Herndon, VA, USA
[b] Dipartimento di Scienza e Tecnologia del Farmaco, University of Turin, Turin, Italy
[c] Department of Pharmacology, University of Illinois at Chicago, Illinois, USA

ARTICLE INFO

Article history:
Received 1 March 2017
Received in revised form 18 July 2017
Accepted 23 July 2017
Available online 25 July 2017

Keywords:
β-cyclodextrin derivatives
Toxin inhibitors
Clostridium perfringens
ε-toxin
Bacillus anthracis
Lethal toxin

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*.

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 et al., 2017). Earlier, a new way of using cyclodextrin derivatives was suggested – utilizing them as direct antimicrobial agents (Karginov et al., 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 et al., 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 et al., 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 et al., 2013); therefore, a great need exists for the development of effective drugs against this toxin.

Since ETX forms heptameric pores in sensitive cells (Miyata et al., 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.

* Corresponding author at: KFBio, LLC, 43136 Fling Ct., Ashburn, VA 20148, USA.
E-mail addresses: vak@kfbllc.com, karginov@gmail.com (V.A. Karginov).
[1] Current address: Naval Medical Research Center, 503 Robert Grant Ave., Silver Spring, MD 20910, USA.
[2] Current address: KFBio, LLC, 43136 Fling Ct., Ashburn, VA 20148, USA.

http://dx.doi.org/10.1016/j.ijpharm.2017.07.070
0378-5173/

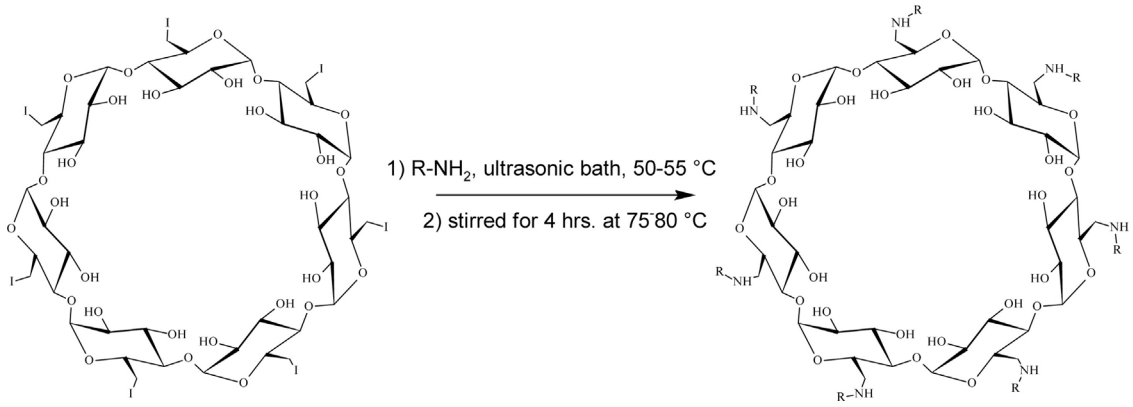

Fig. 1. Synthesis of per-6-deoxy-6-N(alkyl)amino-β-cyclodextrins.

2. Materials and methods

2.1. General description of the synthesis of the cyclodextrin derivatives

The scheme of the synthesis is presented in Fig. 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 min 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 ^{1}H NMR spectrum and it was used without further purification.

Heptakis(6-N-alkylamino-6-deoxy)-β-cyclodextrins were prepared as is summarized in Fig. 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 h 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 min 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 and nuclear magnetic resonance spectroscopy and mass spectrometry analysis of the synthesized compounds are presented in Supplementary data.

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

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 2×10^4 cells/well in 96-well cell culture plates (Becton Dickinson, Franklin Lakes, NJ) and incubated overnight at 37 °C with 5% CO_2. 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 h. 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 A_{590} 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 (Fig. 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),