Symmetry Requirements for Effective Blocking of Pore-Forming Toxins: Comparative Study with α -, β -, and γ -Cyclodextrin Derivatives[†]

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We compared the abilities of structurally related cationic cyclodextrins to inhibit *Bacillus anthracis* lethal toxin and *Staphylococcus aureus* α -hemolysin. We found that both β - and γ -cyclodextrin derivatives effectively inhibited anthrax toxin action by blocking the transmembrane oligomeric pores formed by the protective antigen (PA) subunit of the toxin, whereas α -cyclodextrins were ineffective. In contrast, α -hemolysin was selectively blocked only by β -cyclodextrin derivatives, demonstrating that both symmetry and size of the inhibitor and the pore are important.

Previously, we proposed a novel approach for the discovery of inhibitors of pore-forming toxins that involves the blockage of the pores using molecules with comparable dimensions and the same symmetry as the target pores. It allows for the identification of lead compounds faster and significantly more cheaply in comparison with the existing industry standards. First, this approach was successfully tested on anthrax lethal toxin (LeTx), which plays a key role in anthrax infection. The toxin was disabled by the blockage of the pore formed by protective antigen (PA₆₃), an essential component of anthrax toxin, by rationally designed compounds. Based on the 7-fold symmetry of the PA₆₃ pore, we synthesized and tested cyclic molecules that had 7-fold symmetry using β -cyclodextrin (β -CD) as a starting molecule (Fig. 1). The discovered inhibitors of anthrax toxin were successfully tested *in vitro* and *in vivo* (1–5, 7, 10). The broader applicability of this approach was demonstrated using as targets two other toxins that form transmembrane pores with 7-fold symmetry: α -hemolysin (α -HL) of *Staphylococcus aureus* (2, 11) and ϵ -toxin produced by *Clostridium perfringens* (unpublished data).

To investigate how the structural features of the pore blockers affect their activities, we evaluated the ability of structurally related derivatives of α -, β -, and γ -cyclodextrins to inhibit the cytotoxic activities of anthrax lethal toxin (LeTx) and staphylococcal α -HL as well as to block the ion current through the channels formed by PA₆₃ and α -HL in planar lipid membranes.

The structures of the compounds tested in this study are

presented in Table 1. The synthesis of compounds 8, 9, and 11 to 17 (8, 9) as well as compounds 19 (3) and 22 (2) was described previously. The preparation of compounds 1 to 7, 10, 18, 20, 21, and 23 is presented in the supplemental material. Compounds 1 to 20 were tested for their ability to protect mouse macrophage RAW 264.7 cells from LeTx-induced cell death as described in our earlier publications (2–4). Inhibition of the cytotoxic activity of α -HL was evaluated using a rabbit erythrocyte cell lysis assay as described in reference 2. For some of these compounds, we also evaluated their ability to block the ion current through the pores formed by PA₆₃ and α -HL in planar lipid membranes (2).

Anthrax toxin inhibition. First, we compared the activities of per-6-amino derivatives carrying unmodified hydroxyl groups at positions 2 and 3 (compounds 1 to 3) with the 2,3-methylated ones (compounds 4 to 6; Fig. 1 shows OH group numberings) (Table 1). None of the methylated derivatives displayed toxin-inhibiting activities, while the unmodified compounds 2 and 3 showed activity at low-micromolar concentrations. These data demonstrate the importance of the H-bonding network for the rigidity of the cyclodextrin core, which is destroyed by methylation of the hydroxyl groups at positions 2 and 3.

We also investigated the activities of various α -, β -, and γ -cyclodextrin derivatives carrying the same modifications. Similarly to results obtained earlier (3), the α -cyclodextrin derivatives showed no (compounds 1, 7, 10, and 13) or low (compound 18) LeTx-inhibiting activity. This finding could be explained by their smaller size or by the features of α -CD's structure, which could provide a less favorable spatial orientation of the substituting groups due to the mismatch of the 6-fold symmetry of α -CD and the 7-fold symmetry of the PA₆₃ pore.

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FIG. 1. Schematic illustration of α -, β -, and γ -cyclodextrin molecules in comparison with staphylococcal α -HL channel (left) and anthrax PA (right) prepore. The sizes of cyclodextrin molecules are taken from reference 12.

All the β - and γ -cyclodextrin derivatives with free OH groups at positions 2 and 3 displayed anti-LeTx activity in the micromolar range. When we compared structurally similar derivatives of β - and γ -cyclodextrins, it appeared that in many instances the γ -CDs were more active than the β -CD derivatives (compounds 2 and 3, 8 and 9, 14 and 15, and 16 and 17). That could be related to the recently reported observation of PA₆₃ octameric pores having the same 8-fold symmetry as γ -CD (6). It is interesting also that in the case of the structurally related amino and guanidino derivatives, the latter ones were more active (compounds 2 and 14, 3 and 15, 8 and 16, and 9 and 17).

The ability of several key compounds to block PA channels was tested in channel reconstitution experiments. The results showed (Table 1) that there is a general correlation between the cytotoxicity inhibition and channel blocking activity. Similarly to the results of the cytotoxicity experiments, α -CD derivatives (compounds 1, 13, and 18) exhibited a much lower blocking activity than the β - and γ -CD derivatives (compounds 2, 3, 14, 15, 19, and 20).

α -Hemolysin inhibition. We also evaluated the activities of two α - and γ -CDs (compounds 21 and 23) having the same substituents on the macrocycle's primary side as the β -CD

derivative (compound 22) which inhibits α -HL (2). The compounds were tested using a rabbit erythrocyte assay. Both α - and γ -CD derivatives did not display any activity against α -HL. Similar results were obtained when we compared the compounds' abilities to block the pores formed by α -HL in planar lipid membranes.

The fact that only derivative 22 with the 7-fold symmetry blocked the heptameric α -HL pore is consistent with the idea that a symmetry match between the blocking molecule and the pore is required for effective inhibition. The difference in the activities of γ -CD derivatives against anthrax and staphylococcal toxins could be explained by the recent observation that in contrast to α -HL, PA presumably can form octameric pores in addition to heptameric ones (6). However, the currently available data are ambiguous. For example, our ion channel measurements showed that both β -CD and γ -CD derivatives independently and completely blocked all PA channels in the planar lipid membranes, bringing the transmembrane current to zero. Earlier we reported two types of PA channel insertions (10) that might be attributed to heptameric and octameric channel formation, but the kinetic characteristics of β -CD binding to these channels were indistinguishable. It should be taken into account that in the channel reconstitution experi-

TABLE 1. Activities of the compounds in this study against LeTx or PA (compounds 1 to 20) and α -HL (compounds 21 to 23)

Compound no.	<i>n</i>	R ₁	R ₂ , R ₃	Inhibition of cytotoxicity IC ₅₀ ^a (μM) of LeTx for compounds 1 to 20 and α -HL for compounds 21 to 23	Inhibition of transmembrane conductance IC ₅₀ (nM) of PA for compounds 1 to 20 and α -HL for compounds 21 to 23
1	6	—NH ₂	—H	>100	1,200 ± 300
2	7	—NH ₂	—H	20 ± 9	140 ± 90
3	8	—NH ₂	—H	12 ± 3	170 ± 50
4	6	—NH ₂	—CH ₃	>100	
5	7	—NH ₂	—CH ₃	>100	
6	8	—NH ₂	—CH ₃	>100	
7	6	—NH(CH ₂) ₂ NH ₂	—H	>100	
8	7	—NH(CH ₂) ₂ NH ₂	—H	77 ± 4	
9	8	—NH(CH ₂) ₂ NH ₂	—H	42 ± 4	
10	6	—NH(CH ₂) ₃ NH ₂	—H	>100	
11	7	—NH(CH ₂) ₃ NH ₂	—H	17 ± 1	
12	8	—NH(CH ₂) ₃ NH ₂	—H	41 ± 4	
13	6	—NHC(=NH)NH ₂	—H	>100	2,200 ± 400
14	7	—NHC(=NH)NH ₂	—H	14 ± 2	40 ± 10
15	8	—NHC(=NH)NH ₂	—H	3.6 ± 0.9	32 ± 5
16	7	—NH(CH ₂) ₂ NHC(=NH)NH ₂	—H	29 ± 15	
17	8	—NH(CH ₂) ₂ NHC(=NH)NH ₂	—H	11 ± 2	
18	6		—H	45 ± 13	29 ± 5
19	7		—H	0.8 ± 0.5	0.13 ± 0.10
20	8		—H	5.4 ± 0.8	2.8 ± 1.3
21	6		—H	>100	>5,000
22	7		—H	3.3 ± 2.3	~50
23	8		—H	>100	>5,000

^a IC₅₀, 50% inhibitory concentration.

ments only transmembrane pore blockage is detected. The cell toxicity inhibition could be more complex and also involve inactivation of the prepore. Further studies are required to elucidate the fine mechanisms of the heptameric and octameric pore formations and their relative ratios in the prepores and transmembrane pores as well as the parameters of binding of cyclodextrin derivatives to those forms. To conclude, these data showed that size and conformation as well as the match of the symmetry of a blocking molecule and a pore play important roles in the activity of the inhibitors of pore-forming toxins.

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