Inhibitors of pore-forming toxins

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Introduction

A significant number of bacteria, including the emerging multidrug-resistant “superbugs,” such as Pseudomonas aeruginosa, Clostridium difficile, Staphylococcus aureus, and Escherichia coli, secrete highly potent exotoxins with no antitoxins currently available to disable them. Several bacterial toxins, such as the botulinum neurotoxin (BoNT) of Clostridium botulinum, can be aerosolized and used as biological weapons directly. With some bacterial diseases, such as anthrax, flu-like symptoms appear only after bacteria have proliferated inside the host and have started to produce toxins that ultimately cause death [1]. Even though aggressive antibacterial treatment effectively inhibits Bacillus anthracis propagation, patients can still die because of intoxication [2]. Furthermore, several engineered strains of B. anthracis, which are resistant to the existing antibacterial agents, have already been developed [3,4]. Therefore, the discovery and development of small-molecule antitoxins represent a high-priority task in drug design, medicinal chemistry, and biodefense [4,5]. In the last ten years, a number of biophysical studies have focused on finding ways to disable those bacterial toxins which form ion channels in the host’s cell membranes. This chapter focuses on the existing efforts to design antitoxins that counteract channel-forming bacterial toxins.

Idea of toxin inhibition by channel blockage is suggested by nature

As evolution has progressed, nature has created various neurotoxins, which are small molecule or peptide-based toxins that exclusively target ion-selective channels of excitable cells [6–8]. Humanity has had many interactions with neurotoxins over history, much to its detriment. Regardless of which version of Cleopatra’s self-poisoning is factual, toxin-triggered alteration of channel functioning was likely involved. She could have either suffered from intoxication following blockage of her ligand-gated channels by venom of the Egyptian cobra or from the fatal action of the chloride channel inhibitor extracted from cicuta.
These days, due to research on the biological functions of neurotoxins, these compounds are used not only to understand the structural and functional aspects of ion channels of excitable membranes, but also to advance the pharmaceutical approaches to use these toxins in different therapeutic applications. Contrariwise, among the great variety of virulence factors secreted by different organisms, including bacteria, there is a significant group of toxins, which instead of blocking the host ion-selective channels, form ion-conductive pores in cell membranes. For most of these toxins, no effective blockers have been developed and approved for animal or human use. However, one of the modern ideas to target the bacterial exotoxins is obvious and simple. Following nature, which generated pore-blocking toxins, we can rationally design powerful antitoxin agents that will specifically block the conductive pathways of the pore-forming virulence factors. This idea has already seen its medical application within the past 50 years. In 1965, amantadine, the small-molecule blocker of the tetrameric proton-selective M2 channel from the viral envelope of influenza A virus [9–11] had been approved for human use. Despite the developed resistance of the virus to amantadine, the M2 channel is still one of the main targets for virtual screening and rational design of prospective drugs to treat influenza. A number of different viroporins, including p7 of hepatitis C virus and viral protein U (Vpu) of HIV-1, were also explored as potential targets for the development of pore-blocking agents. Therefore, the approach originally created by nature to modify the channel’s functions by the selectively acting pore-blocking agents enjoys well-deserved attention. Despite not being able to replicate the same evolutionary path which nature has previously taken, it is now possible to use modern-day single-channel and single-molecule biophysical methodologies to investigate channel-forming toxins and to rationally design their potential inhibitors by exploring the roles of various interactions involved in channel-facilitated transport.

**Pore-forming bacterial toxins and their inhibitors**

*Interactions with solute define channel transport properties*

Transport properties of a channel are governed by its interactions with solute molecules. In addition to Coulomb, van der Waals, hydrogen bonding, and other short-range forces, an important role belongs to entropic interactions. Indeed, ion- and metabolite-specific channels restrict translational freedom of solutes that they transport by confining the molecules within their water-filled pores. In addition, the geometry of practically every known channel pore is not that of a regular cylinder, but is often characterized by constrictions along the channel axis. During the past decade, an analytical approach was developed [12–18], which allows one to calculate the major transport parameters such as the probability and characteristic times of solute translocation. The approach is based on the description of the solute particle diffusion in the channel in terms of the particle Green’s function $G(x,t;x_0)$. It is defined as the probability density to find the particle at point $x$ at time $t$ if it was at $x_0$ at $t = 0$ and
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has not escaped from the channel during that time. The Green’s function obeys the Smoluchowski equation:

\[
\frac{\partial G(x,t;x_0)}{\partial t} = \frac{\partial}{\partial x} \left[ D_{ch}(x) \exp(-\beta U(x)) \frac{\partial}{\partial t} \left\{ \exp(\beta U(x))G(x,t;x_0) \right\} \right], \tag{38.1}
\]

with the initial condition \(G(x,0;x_0) = \delta(x - x_0), \quad 0 < x < L,\) and the radiation boundary conditions at the channel openings at \(x = 0\) and \(x = L\)

\[
\begin{align*}
\frac{\partial}{\partial x} \left[ G(x,t;x_0) \exp(\beta U(x)) \right] \bigg|_{x=0} &= \frac{k_0}{D_{ch}(0)} G(0,t;x_0) \exp(\beta U(0)), \\
\frac{\partial}{\partial x} \left[ G(x,t;x_0) \exp(\beta U(x)) \right] \bigg|_{x=L} &= \frac{k_L}{D_{ch}(L)} G(L,t;x_0) \exp(\beta U(L)).
\end{align*} \tag{38.2}
\]

Here, \(\beta = 1/(k_B T),\) with \(k_B\) and \(T\) having their usual meanings of the Boltzmann constant and absolute temperature, respectively; and \(D_{ch}(x)\) is the particle diffusion coefficient inside the channel. The potential of mean force \(U(x)\) includes, in the general case, contributions not only from actual physical forces, but also entropic potential accounting for the varying channel geometry. The rate constants \(k_0\) and \(k_L\) relate the one-dimensional (1D) problem described by Eqs. (38.1) and (38.2) with the three-dimensional (3D) case of interest by characterizing the escape efficiency. For the channel of a complex (including asymmetric) shape, but with identical circular openings of radius \(R\) on both ends, these rate constants are [12–14]:

\[
k_0 = k_L = k = \frac{4D_b}{\pi R}, \tag{38.3}
\]

where \(D_b\) is the particle diffusion coefficient in the bulk.

In the general case, it is impossible to solve Eqs. (38.1)–(38.3) analytically; however, the most important characteristics of the particle dynamics in the channel can be calculated explicitly. Among them, the particle translocation probability is of key interest. In the absence of the external fields, \(U(0) = U(L),\) and with Eq. (38.3) satisfied, the probabilities to translocate from the left to the right, \(P_{tr}(0),\) and from the right to the left, \(P_{rt}(L),\) are equal to each other and are given by [13]

\[
P_{tr}(0) = P_{rt}(L) = \frac{1}{2 + \frac{4D_b}{\pi R} \int_0^L \frac{\exp(\beta U(x))}{D_{ch}(x)} \, dx}. \tag{38.4}
\]

It is seen that with the increasing attractive interactions (that is, a deep potential well, \(-\beta U(x) \gg 1),\) the translocation probability increases to its maximal value of \(1/2\) for the diffusion in the absence of the external driving force.
Other important parameters are different characteristic times spent by the particle in the channel. Among them are the mean translocation time, return times (for a particle which enters and leaves the channel from the same side), and lifetimes of the particle in the channel. For the translocation time, it was found, surprisingly, that it is direction-independent,

\[ \tau_{tr} = \tau_{tr}(L) = \tau_{tr}, \]

and given by [14]

\[
\tau_{tr} = \int_0^L \left[ 1 + k_0 \int_y^L \frac{\exp(-\beta U(y))}{D_{ch}(y)} dy \right] \left[ 1 + k_L \exp(-\beta \Delta U) \int_y^L \frac{\exp(-\beta U(x))}{D_{ch}(y)} dx \right] \exp(-\beta U(x)) dx, \tag{38.5}\]

where \( \Delta U = U(L) - U(0) \). Moreover, not only the mean value, but also the probability density of the translocation time is direction independent [18]. However, the mean lifetimes in the channel, depend on the side from which the particle enters the channel. Even for \( U(0) = U(L) \), they are still side dependent [14]:

\[
\tau(0) = \int_0^L \left[ 1 + k_0 \int_y^L \frac{\exp(-\beta U(y))}{D_{ch}(y)} dy \right] \exp(-\beta U(x)) dx, \tag{38.6}\]

\[
\tau(L) = \int_0^L \left[ 1 + k_L \int_y^L \frac{\exp(-\beta U(y))}{D_{ch}(y)} dy \right] \exp(-\beta U(x)) dx. \tag{38.6}\]

Eqs. (38.5) and (38.6) give the mean times for arbitrary dependences of the potential of mean force, \( U(x) \), and particle diffusion coefficient, \( D_{ch}(x) \), on the particle coordinate in the channel, \( x \). More general formulas for the translocation probability and characteristic times are available in [14,18].

For a simplified case of a symmetric cylindrical channel with a rectangular potential well of depth \( U_0 \) occupying the entire channel and a position-independent diffusion coefficient \( D_{ch}(x) = D_{ch} \), the expressions for the translocation probability and average lifetime in the channel simplify and take the form

\[
P_{tr} = \frac{1}{2 + \frac{4D_{ch}L}{\pi D_{ch}R} \exp(-\beta U_0)}, \tag{38.7}\]

and

\[
\tau = \frac{\pi RL}{8D_{br}} \exp(\beta U_0). \tag{38.8}\]
Using this simplification, and introducing particle-particle repulsive interaction as a requirement that the channel can be occupied by only one particle at a time, it was shown that the particle flux through the channel is [15–18]

\[
J = \frac{2D_b R (c_1 - c_2)}{1 + \frac{\pi R^2 L (c_1 + c_2)}{2} \exp(\beta U_0)} \left[ 1 + \frac{2D_b L}{\pi D_{ch} R} \exp(-\beta U_0) \right].
\]  
(38.9)

where \(c_1\) and \(c_2\) are the particle concentrations on the two sides of the membrane. As expected, the flux is a nonmonotonic function of the well depth. Indeed, at the well depth that maximizes the flux, there should be a compromise between sufficiently high translocation probability [Eq. (38.7)], and a not-too-long particle lifetime in the channel [Eq. (38.8)]. The concentration-dependent optimal depth is given by

\[
U_{opt} = \frac{k_B T}{2} \ln \left[ \frac{4D_b}{\pi^2 D_{ch} R^4 (c_1 + c_2)} \right].
\]

Interpretation of this result is quite straightforward: If the interaction is not strong enough (that is, below the optimal level), the particle tends to leave the channel from the same side it entered. For this obvious reason, the flux reduces. On the other side of the optimal, overly strong interactions lead to channel blockage. In this case, the particle spends too much time in the channel, blocking the channel for other particles. The flux through the channel at the optimal versus too strong or too weak interactions is shown in Figure 38.1.

**Practical examples of the inhibition of channel-forming bacterial toxins**

Successful realization of numerous *in vitro* biophysical studies with purified pore-forming bacterial toxins has been made possible due to the unique ability of these proteins to exist both in a stable, water-soluble conformation and in a membrane-surrounded conformation. The pore-forming bacterial toxins or the toxins’ pore-forming components can form stable ion conductive channel structures in the model bilayer membranes, which lack any extracellular receptors normally required for their *in vivo* binding. X-ray crystallography has provided important details of many protein structures and functions, and the protein-recombinant technology has allowed for a significant number of different mutant molecules to be synthesized, in which key amino acids were mutated in order to uncover their critical roles in the channel function. Single-molecule measurements performed with model bilayer methods or using computer-aided modeling techniques allowed the exploitation of unique features of the target biomolecules, small-molecule or macromolecule drug candidates and of exact physical forces that govern their interactions. The abovementioned approaches
have ultimately served to initiate the development of the area of research that we now refer to as the targeted design of effective channel-blocking agents.

Bacterial toxins are separated into two functionally different groups: endotoxins and exotoxins [19]. Endotoxins, such as lipopolysaccharides, are components of the outer membrane of Gram-negative bacteria. Exotoxins, which are the subject of this chapter, are proteins secreted by a number of Gram-positive and Gram-negative bacteria. These toxins effectively act on host eukaryotic cells at a considerable distance from the pathogen itself. There are several ways to further classify these channel-forming proteins. The classification is often based on the pathogen source (such as clostridial toxins [20]), on the mechanism of their cytotoxicity (such as membrane-damaging exotoxins [21] or ADP-ribosylating exotoxins [22]), or on their distinctive structural forms (such as α-helical and β-barrel channel-forming toxins [21,23]). For the task discussed here, we chose to use a combined structural/functional classification to sort the bacterial toxins into the following two categories.

**AB-type bacterial toxins.** Several exotoxins act in the cytosol of mammalian cells, where they enzymatically modify their specific intracellular substrates. Many of these toxins are secreted as either single-chain proteins containing at least two functional domains [namely, the receptor binding B domain and the active/enzymatic A domain (classical AB-type toxins)]; or as two (or three in the case of anthrax toxin) individual nonlinked binary toxin subunits, a binding/translocation A component and enzymatic/
active B component (binary AB-type toxins) [24]. The binding B domain of the AB-type toxins docks to a receptor on the host cell surface and often mediates intracellular transport of the toxin, whereas the active/enzymatic A domain alters specific substrates in the cytosol. In particular, following the receptor-mediated endocytosis, the B components of the binary bacterial toxins secreted by several pathogenic species of *Bacillus* and *Clostridium* not only provide the binding site for the A components, but also form oligomeric transmembrane channels as well. These channels are suggested to serve as active translocation pathways for the transport of the A component.

Membrane-perforating bacterial toxins. In contrast to the AB-type toxins, which act in the cytosol of mammalian cells, membrane-perforating bacterial toxins directly compromise host cells by inducing uncontrollable leakages of ions, water, and water-soluble metabolites. Membrane-perforating bacterial toxins represent more than one-third of the described protein toxins [21,23]. These agents act by incorporating into host membranes to form large, water-filled, ion-permeable pores. The membrane-perforating bacterial toxins allow for the flow of ions down their electrochemical gradients, which causes ion imbalance and disruption in the membrane potential. This elicited disruption of membrane potential in turn may further confer a compromise of the plasma membrane barrier functionality. To emphasize the mechanism of the membrane-perforating action, these toxins are often referred to as pore-forming toxins (PFTs). Among typical examples of the membrane-perforating bacterial toxins are α-hemolysin (α-HL; occasionally called α-toxin or α-haemolysin) and other PFTs of *S. aureus* and epsilon toxin (ETX) of *Clostridium perfringens*. Note: channel-forming proteins play quite distinct roles in the cell intoxication by membrane-perforating and AB-type bacterial toxins. However, for the practical purpose of this chapter, we will use the term channel-forming (or pore-forming) bacterial toxins when referring to any of these types of toxins.

Pore blockers to target bacterial binary AB-type toxins

The current studies searching for effective therapies against the binary bacterial toxins primarily focus on targeting the anthrax toxin (recently reviewed in [25]). This situation is entirely determined by the terrorist 9/11 attack and the fatal consequences of intentional dissemination of *B. anthracis* spores via the so-called “anthrax letters” of 2001. At the same time, the similarities between channel-forming B components of the anthrax toxin and clostridial binary toxins were used to explore these channels as specific universal targets for development of new broad-spectrum antitoxins against the *Bacillus* and *Clostridium* pathogenic species alike.

Anthrax toxin of *B. anthracis*

Recent developments made in the understanding of anthrax toxin intracellular translocation and mechanism of its action are quite noteworthy. For the purpose of this chapter, we will discuss only the main aspects of these developments and direct readers to the corresponding Chapter 13 of this book, written by Stephen
Leppla and colleagues. The tripartite anthrax toxin, as a member of the bacterial AB exotoxin family, is made of three individual proteins that self-assemble at the surface of the targeted cell. It consists of two enzymatic A components: lethal factor (LF), a Zn-metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPKK) inducing the cell death of macrophages, and edema factor (EF), a Ca²⁺- and calmodulin-activated adenylyl cyclase [26–29], and one shared translocation/binding B-component (83 kDa protective antigen, or PA). It is important to mention that the name protective antigen originates from its long-lasting use as an active component in a vaccine against anthrax, and it does not refer to any protective properties of this protein during anthrax toxin infection. PA, LF, and EF, being individually nontoxic, associate to form “classic” AB-type binary toxins [24]: lethal toxin (LT = LF+PA) and edema toxin (ET = EF+PA), which, together with a phagocytosis-inhibiting poly-D-glutamic acid capsule, are responsible for the anthrax symptoms and lethality.

The anthrax toxin internalization process involves several steps. First, the full-length binding component PA (PA₈₃) binds to the cellular CMG2 and TEM8 receptors and, after being cleaved by an extracellular furin protease to a 63-kDa form (PA₆₃), goes through oligomerization to form heptameric [30] and/or octameric [31] ring-shaped prepores. The formation of the prepore induces three [32] or four [31] binding sites for LF, EF, or both at the interface of two adjacent PA₆₃ molecules. The formation of the oligomeric prepores also generates a receptor-mediated signaling pathway that triggers endocytosis of the oligomeric anthrax toxin complexes [33]. After endocytosis of the complexes occur, the acidic environment of the endosomes causes substantial structural changes of the oligomeric PA₆₃ prepore. These changes lead to the oligomer insertion into the endosomal membrane, where it forms a cation-selective ion channel [34]. The protein wall of the oligomeric PA₆₃ forms a single tunnel which is a water-filled pore connecting aqueous solutions on both sides of the endosomal membrane. The elongated mushroom-like (of 125 Å diameter with a 70-Å-long cap and 100-Å-long stem) (PA₆₃)₇ structures were detected by negative-stain electron microscopy [35].

After the channel formation, PA believed to act as an effective translocase which, using the proton gradient across the endosomal membrane (pH_{endosome} < pH_{cytosol}), unfolds and translocates LF and EF inside the cell [36–41]. Molecular details of the PA₆₃ channels acting as a translocase have emerged due to the pioneering lipid bilayer measurements performed by both Finkelstein’s and Collier’s groups and, more recently, by Krantz’s group (reviewed in [42–44]). LF and EF translocation through the PA channel was monitored by recording the resumption of ion current, originally decreased by channel blocking by LF/EF after LF/EF was transported. The translocation of LF was initiated by its N-terminus entering the PA₆₃ channel [36] and driven through by either the transmembrane potential [37] or by the proton gradient [45] across the bilayer membrane. The enzymatic subunit transport across the PA₆₃ channel (and consequently its toxicity) were shown to be considerably inhibited when the phenylalanine residue at position 427 was mutated [38].

The seven (or eight) F427 residues are thought to form a narrow constriction zone inside the pore lumen (Φ-clamp), which acts as a translocase site critical for the
active substrate uptake. To answer the fundamental question regarding the origin of the protein translocation driving force, the authors suggest that the PA$_{63}$ symporter achieves protein translocation using a tandem of two synergistic Brownian ratchets: the $\phi$-clamp ratchet, promoting the substrate unfolding, and the charge-state ratchet, which biases the entry rates of the substrates into the pore. A conceptually different model of anthrax toxin translocation was recently suggested by Nablo and colleagues [46]. The model proposes that instead of the active components being threaded through the pore, anthrax toxin complexes (namely, LF or EF bound to the PA$_{63}$ channel) rupture membranes. Once in the cytosol, LF and EF perform their catalytic actions. As mentioned before, LF is a Zn-metalloprotease that cleaves MAPKK [26,47] and NLrp1 [48], which effectively works to disturb host cell signal transduction. EF is a calmodulin-dependent adenylyl cyclase that increases the cyclic adenosine monophosphate (cAMP) level in the targeted cells, contributing to systemic dispersal of $B.\\text{anthracis}$ within the Eukaryotic host [49]. The key tissue targets responsible for the toxic effects of LT and ET were identified recently [50]. LT and ET were reported to target the cardiovascular system and liver, respectively.

**Clostridial binary toxin B subunits are close orthologs of the PA of anthrax**

Several pathogenic species of Clostridia secrete binary exotoxins, which are closely related to the anthrax toxin of $B.\\text{anthracis}$ (see Chapter 14 written by Barth, Stiles, and Popoff, for details). Among those toxins are C2 toxin of $C.\\text{botulinum}$, iota toxin of $C.\\text{perfringens}$, $C.\\text{difficile}$ toxin (CDT), and $\text{Clostridium spiroforme}$ toxin (CST). Both striking similarities and prominent differences were reported between the anthrax and clostridial binary toxins. While the anthrax toxin is a tripartite toxin made by the two enzymatically active A components and one binding/translocation B component, clostridial binary toxins are made of two components only [24,42]. In contrast to that of the anthrax toxin, the enzymatic A components of clostridial binary toxins act through mono-ADP-ribosylation of G-actins, causing a complete destruction of the actin cytoskeleton and caspase-dependent cell death [19,51–59]. X-ray crystallography of both the PA component of anthrax toxin and the B component of the C2 toxin (C2II) have shown four distinct domains involved in cellular receptor binding, oligomerization, pore formation, and A component binding [30,60]. The B components of anthrax and clostridial binary toxins share high degrees (from 27% to 38%) of amino acid homology. The amino acid sequence similarity between the B components is primarily localized within the central domains: domain II, responsible for pore formation and A component translocation; and domain III, which is important for the oligomerization of monomers. There is relatively little homology of domain I, which is responsible for A component binding; and domain IV, which is important for cellular receptor binding. This finding is explained by the fact that PA and the binary toxin B components have evolved to dock significantly different enzymatic A components and to bind to different cellular receptors. Following proteolytic
activation, the truncated B components of binary toxins form ring-shaped oligomers, called *prepores*, on the surface of eukaryotic cells or in solution [61]. In contrast to the PA oligomers, formation of eightfold symmetrical C2IIa and Ib oligomers was not reported. A model structure of the C2 toxin’s B component (C2IIa)\(_7\) prepore was constructed based on the corresponding structural assembly of the (PA\(_{63}\))\(_7\) prepore [30,60], for which the X-ray structure was solved. The cellular uptake mechanisms of the anthrax and clostridial toxins are related, though with several distinctions, and are described in detail elsewhere [19,24]. Similarly to the anthrax toxins, the cell-bound C2I/C2IIa, Ia/Ib, and CDTa/CDTb complexes are internalized by receptor-mediated endocytosis [62–65]. After endocytosis, these complexes reach endosomal vesicles where the A components translocate across the endosomal membranes into the cytosol, possibly using the C2IIa and Ib pores as translocation corridors [61,65–70], \textit{in vitro} model bilayer experiments have showed that PA\(_{63}\) is able to bind and translocate His-tagged C2I, while C2II binds, but does not translocate LF and EF [71].

Up to now, well-defined and stable ion channels formed in bilayer lipid membranes were reported for the B components of C2 and iota toxins. In mildly acidic conditions (pH < 6.6), the B components of C2 and iota toxins form ion-permeable, cation-selective ion channels [34,66,67,72]. Similarly to PA, the phenylalanine clamp (ϕ-clamp), preserved in position 428, was found to catalyze the unfolding and translocation of the C2I components across the membrane [73,74]. The phenylalanine residue at the proximate position is also conserved in Ib [74]. Interestingly, the current noise characteristics of the PA\(_{63}\), C2IIa, and Ib channels are alike, as the voltage-independent flickering of the current between open and completely closed states was detected in high-resolution recordings [66,72,75–77]. This flickering was characterized by complex non-Markovian kinetics giving a 1/f-type shape of the current power spectra [77]. At the same time, the F427A mutant of PA\(_{63}\) was shown to lack this complex conductance fluctuation behavior. Note: the 1/f fast flickering between the open and completely closed states is unique for the family of channel-forming B components of binary toxins; α-HL and many other β-barrel pore-forming proteins, which similarly to PA\(_{63}\), C2IIa, and Ib form heptameric channels, do not show this type of fluctuations [78,79]. In addition to this voltage-independent flickering, a strong voltage-dependent "gating" of the PA\(_{63}\), C2IIa, and Ib channels, which was earlier observed with many β-barrel channels, including artificial ones [80–82], was also reported.

**Small-molecule cationic pore blockers**

Multiple compounds, which are charged positively at mildly acidic pH values, were shown to interact with PA\(_{63}\), C2IIa, and Ib channels incorporated into planar bilayers [38,67,72,74,75,83,84] (Figure 38.2). The ability of tetraalkylammonium ions to translocate through PA\(_{63}\), reversibly blocking the K\(^+\) current [76,85,86], was used to estimate the minimum physical size of the PA\(_{63}\) pore lumen to be about 12 Å. This value was subsequently confirmed with several independent methods [35,87,88] and was used for the rational design of anthrax toxin PA\(_{63}\)-blocking inhibitors [89]. It was shown that the tetraalkylammonium binding site was accessible from both cis- and trans- sides of a bilayer chamber, which often indicates the ability of ions...
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Moreover, the tetraalkylammonium binding time as a function of transmembrane voltage had a pronounced maximum, which is also representative of the permeable versus impermeable blocking molecules [90–93]. An ion of tetraheptylammonium as large as about 12 Å could translocate through PA63, whereas the entry rate of tetrahexylammonium and tetraheptylammonium ions [86,94] was impetuously decreased. The PA 63/tetraalkylammonium ion binding reaction was characterized as a diffusion-controlled interaction [86]. In accordance with these findings, cells were reported to be protected from the binary toxin action by several weak bases [94–96]. The antimalarial drug chloroquine blocked the PA63 (K_D^PA = 0.51 μM in 0.1 M of KCl) and C2IIa (K_D^C2IIa = 10 μM in 0.15 M of KCl) channels in vitro and prevented translocation of active C2I moiety of the C2 toxin across the host cell membrane when studied with intact cultured cells. Only a weak current inhibition by chloroquine was reported with the iota toxin’s B moiety, Ib, in the planar bilayers (K_D^Ib = 0.22 mM in 0.1 M of KCl), which was not sufficient to protect cells from iota toxin–induced intoxication [72]. To determine the nature of the blocker/pore-binding reaction, Benz and colleagues investigated parameters of the binding kinetics of chloroquine and related compounds to the PA63 and C2II channels using the planar bilayer technique [74,75,83]. The authors showed that addition of...
of the blocker ligands to membrane-bathing solutions leads to an ion current noise increase with the spectral density of the Lorentzian type that is typical for a simple binding-site model [75, 83]. The binding reaction on-rate, which characterizes the frequency of the blockage events, was largely a function of the bathing solution’s ionic strength. This type of behavior is often attributed to ion-ion electrostatic interactions involved in blocker binding. The binding affinity of the positively charged molecule decreases in the order PA63 > C2IIa ≫ Ib. This finding was explained by a decreasing number of the potential binding sites formed by the negatively charged amino acid residues in the cis entrance of these channels. The binding reaction off-rate, which characterizes the residence time of the compound in the pore, was dependent on the structural features of the blocker molecules. Thus, the PA63 and C2IIa ion current inhibition by chloroquine was highly asymmetrical when the ligand was added either to the cis- or the trans-sides of the membrane. The trans-side addition resulted in a significantly decreased binding affinity. However, the action of another blocker, an antipsychotic drug fluphenazine, was quite symmetrical. We emphasize the side of the blocker’s action because channel insertion into the model bilayer membranes is usually highly unidirectional. It is expected that the “cap” opening of the pores (assuming a mushroom-like shape for both PA63 and C2IIa) faces the cis-side solution, which is the side of protein addition, and the membrane-inserted β-barrel stem opening faces the trans-side.

In addition to the negatively charged amino acid residues, the ϕ-clamp was discussed as a potential site for binding of the PA63 pore blockers [38]. The authors examined a preselected library of 35 available cationic quaternary ammonium and phosphonium ion compounds in order to test their blocking activity against the PA63 channel (Table 38.1). Interestingly, the mutations in the ϕ-clamp were shown to profoundly affect the binding affinity of hydrophobic cations, such as tetrabutylammonium (TBA); TBA’s affinity to F427A mutant channels was about 4000 times lower compared with that of the wild type. The authors hypothesized that the ϕ-clamp site, being hydrophobic, may directly bind the hydrophobic cations such as TBA. The pore-blocking mechanism by TBA would include cation-π interactions between aromatic residues interacting with cations through their delocalized negative π-electron cloud. Indeed, among the 35 compounds studied, the more hydrophobic ones possessed higher inhibitory activity against the PA63 channel. In particular, a TBA analog of a similar size carrying hydrophilic amide and ester groups was 140-fold less active in inhibiting PA63 current, whereas tetraphenylphosphonium was 160-fold more active compared to TBA. In general, the ϕ-clamp preferred aromatic moieties by 0.7 kcal/mol per aromatic ring with the most effective compounds showing KD < 1 μM [38]. More important, the nM-range binding affinity toward the channel was observed with the compounds carrying three or four aromatic rings. The authors suggested that the ϕ-clamp could be responsible for nonspecific hydrophobic interactions, although its negative π-clouds could also contribute through aromatic-aromatic, π-π, and cation-π interactions. The effect was significantly diminished with the F427A mutants, showing potential importance of exploring this binding site in the development of channel-blocking drugs.

These reports, taken together, indicate that the potential effective blockers against channel-forming components of the binary toxins should be designed focusing special
### Table 38.1 Small-molecule cationic blockers of PA₆₃ channel conductance

<table>
<thead>
<tr>
<th>Number</th>
<th>Cationic compounds</th>
<th>Structure</th>
<th>IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tetramethylammonium</td>
<td><img src="image" alt="Tetramethylammonium" /></td>
<td>1.6 mM</td>
</tr>
<tr>
<td>2</td>
<td>Tetraethylammonium</td>
<td><img src="image" alt="Tetraethylammonium" /></td>
<td>224 μM</td>
</tr>
<tr>
<td>3</td>
<td>Tetrapropylammonium</td>
<td><img src="image" alt="Tetrapropylammonium" /></td>
<td>350 ± 10 nM</td>
</tr>
<tr>
<td>4</td>
<td>Tetrabutylammonium</td>
<td><img src="image" alt="Tetrabutylammonium" /></td>
<td>7.3 ± 0.2 μM</td>
</tr>
<tr>
<td>5</td>
<td>Tetrapentylammonium</td>
<td><img src="image" alt="Tetrapentylammonium" /></td>
<td>2 μM</td>
</tr>
<tr>
<td>6</td>
<td>Tetrahexylammonium</td>
<td><img src="image" alt="Tetrahexylammonium" /></td>
<td>3.8 ± 0.3 μM</td>
</tr>
<tr>
<td>7</td>
<td>(2-acetylamino-2,2-bis-ethoxycarbonyl-ethyl)-trimethyl-ammonium</td>
<td><img src="image" alt="2-acetylamino-2,2-bis-ethoxycarbonyl-ethyl" /></td>
<td>1 ± 0.1 mM</td>
</tr>
</tbody>
</table>

**Small aromatic cationic compounds [38]**

<table>
<thead>
<tr>
<th>Number</th>
<th>Cationic compounds</th>
<th>Structure</th>
<th>IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Tetrapheny phosphonium</td>
<td><img src="image" alt="Tetrapheny phosphonium" /></td>
<td>46 ± 2 nM</td>
</tr>
<tr>
<td>9</td>
<td>Chloroquine</td>
<td><img src="image" alt="Chloroquine" /></td>
<td>510 ± 30 nM</td>
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(Continued)
<table>
<thead>
<tr>
<th></th>
<th>Cationic compounds</th>
<th>Structure</th>
<th>IC\textsubscript{50}</th>
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<tbody>
<tr>
<td>10</td>
<td>Quinacrine</td>
<td><img src="image" alt="Structure" /></td>
<td>60 ± 5 nM</td>
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<tr>
<td>11</td>
<td>Benzyltriphenylphosphonium</td>
<td><img src="image" alt="Structure" /></td>
<td>110 ± 30 nM</td>
</tr>
<tr>
<td>12</td>
<td>Butyltriphenylphosphonium</td>
<td><img src="image" alt="Structure" /></td>
<td>88 ± 5 nM</td>
</tr>
<tr>
<td>13</td>
<td>Isoamyltriphenylphosphonium</td>
<td><img src="image" alt="Structure" /></td>
<td>35 ± 6 nM</td>
</tr>
<tr>
<td>14</td>
<td>Methyltriphenylphosphonium</td>
<td><img src="image" alt="Structure" /></td>
<td>370 ± 60 nM</td>
</tr>
</tbody>
</table>

**Azolopyridinium salts [97]**

<table>
<thead>
<tr>
<th></th>
<th>Cationic compounds</th>
<th>Structure</th>
<th>IC\textsubscript{50}</th>
</tr>
</thead>
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<tr>
<td>15</td>
<td>HA 486</td>
<td><img src="image" alt="Structure" /></td>
<td>27 μM</td>
</tr>
<tr>
<td>16</td>
<td>HA 1383</td>
<td><img src="image" alt="Structure" /></td>
<td>1.3 μM</td>
</tr>
<tr>
<td>17</td>
<td>HA 1568</td>
<td><img src="image" alt="Structure" /></td>
<td>2.7 μM</td>
</tr>
</tbody>
</table>
attention on both the negatively charged amino acid residues in the channel’s lumen and on the \( \phi \)-clamp. Indeed, when potential binding sites for cationic chloroquine were investigated with the C2IIa channel [74], it turned out that not only does the mutation of the negatively charged amino acids lead to a significant decrease in their affinity for binding chloroquine and its analogs, but the \( \phi \)-clamp mutations (F428A, F428D, F428Y, and F428W) do so as well. It is thought that the \( \phi \)-clamp is preserved within the C2II at the F428 position. Thus, the residues Glu399, Asp426 (probably located in the vestibule near the channel entrance), and Phe428— but not Glu272, Glu280, Asp341, or Glu346— were important for interacting with the C2IIa channel blockers, such as chloroquine and 4-aminoquinolones. The F428A mutation’s effect was the strongest, increasing \( K_D \) values and therefore weakening the binding by a factor of almost 400. These findings show that the substances carrying both the positively charged and the bulky hydrophobic aromatic groups could represent lead compounds suitable for further rational modifications as binary toxin inhibitors. A group of heterocyclic azolopyridinium salts was recently examined to probe their inhibitory activity against PA63 and C2IIa components in the model bilayers and their protective effect against anthrax and C2 toxins in cell assays [97, 98]. Several of these compounds, while being active in the low-micromolar concentrations \( \text{in vitro} \), were fully protective against toxin action in cell assays and had only negligible cytotoxic effects. Interestingly, the authors confirm that the on-rate for compound binding \([1.2 \cdot 10^8 \text{l/(M·s)} – 1.8 \cdot 10^8 \text{l/(M·s)}]\) in 0.15 M of KCl does not vary significantly with the ligand’s structure, being close to that of diffusion-controlled processes. In contrast, the off-rate showed significantly higher variations, increasing when both the size of the ligands and the number of aromatic groups decreased. The highest binding affinity was observed for salts that had a nitroxyl group bound to an aromatic ring [97].

**Polyvalent cationic pore blockers**

A significant increase in the activity of the cationic antitoxins was achieved using a rational design of polyvalent compounds [89] (Figure 38.2). Attaching multiple functional groups onto an inert scaffold was described as an effective emergent drug design approach [99, 100]. If designed and constructed appropriately, the multiligand molecules show an additive or cooperative affinity toward multiple binding sites that is dramatically increased compared with that of a single functional group interacting with a single binding site [99]. A number of bacterial protein toxins have recently been successfully inhibited by novel synthetic polyvalent agents (reviewed in [101]). The important step in design and optimization of the polyvalent inhibitors is to search for a suitable scaffold to attach the ligands, which previously revealed some promising activity against the target [102]. In targeting the binary anthrax and clostridial toxins, these active ligands are represented by a wide variety of positively charged and aromatic groups. This led researchers to a design of synthetic tailor-made cationic 7-positively charged blockers based on a sevenfold symmetrical \( \beta \)-cyclodextrin (7+\( \beta \)CD) core (reviewed in [103]) (Table 38.2). The designed compounds have shown
Table 38.2 Polyvalent cationic cyclodextrin blockers of PA$_{63}$ channel conductance

<table>
<thead>
<tr>
<th>Number</th>
<th>Cyclodextrin</th>
<th>$R_1$</th>
<th>$R_2$, $R_3$</th>
<th>Inhibition of Conductance $IC_{50}$, nM</th>
<th>Inhibition of Cytotoxicity $IC_{50}$, $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hepta-6-aminoalkyl $\beta$-cyclodextrin derivatives [104]</td>
<td>$\beta$</td>
<td>$-\text{NH}_2$</td>
<td>$-\text{H}$</td>
<td>$140 \pm 90$</td>
</tr>
<tr>
<td>2</td>
<td>$\beta$</td>
<td>$-\text{S(}\text{CH}_2\text{)}_2\text{N}_2\text{NH}_2$</td>
<td>$-\text{H}$</td>
<td>$3.5 \pm 0.9$</td>
<td>$7.8 \pm 2.4$</td>
</tr>
<tr>
<td>3</td>
<td>$\beta$</td>
<td>$-\text{S(}\text{CH}_2\text{)}_3\text{N}_2\text{NH}_2$</td>
<td>$-\text{H}$</td>
<td>$0.57 \pm 0.39$</td>
<td>$2.9 \pm 1.0$</td>
</tr>
<tr>
<td>4</td>
<td>$\beta$</td>
<td>$-\text{S(}\text{CH}_2\text{)}_4\text{N}_2\text{NH}_2$</td>
<td>$-\text{H}$</td>
<td>$1.1 \pm 0.5$</td>
<td>$5.1 \pm 2.4$</td>
</tr>
<tr>
<td>5</td>
<td>$\beta$</td>
<td>$-\text{S(}\text{CH}_2\text{)}_5\text{N}_2\text{NH}_2$</td>
<td>$-\text{H}$</td>
<td>$3.8 \pm 1.0$</td>
<td>$7.5 \pm 2.4$</td>
</tr>
<tr>
<td>6</td>
<td>$\beta$</td>
<td>$-\text{S(}\text{CH}_2\text{)}_6\text{N}_2\text{NH}_2$</td>
<td>$-\text{H}$</td>
<td>$0.97 \pm 0.38$</td>
<td>$0.6 \pm 0.3$</td>
</tr>
<tr>
<td>7</td>
<td>$\beta$</td>
<td>$-\text{S(}\text{CH}_2\text{)}_7\text{N}_2\text{NH}_2$</td>
<td>$-\text{H}$</td>
<td>$4.6 \pm 3.2$</td>
<td>$1.9 \pm 1.1$</td>
</tr>
<tr>
<td>8</td>
<td>$\beta$</td>
<td>$-\text{S(}\text{CH}_2\text{)}_8\text{N}_2\text{NH}_2$</td>
<td>$-\text{H}$</td>
<td>$2.4 \pm 0.95$</td>
<td>$0.3 \pm 0.1$</td>
</tr>
<tr>
<td>9</td>
<td>$\beta$</td>
<td>$-\text{S(}\text{CH}_2\text{)}_9\text{N}_2\text{NH}_2$</td>
<td>$-\text{H}$</td>
<td>$27.0 \pm 17.0$</td>
<td>$2.6 \pm 0.7$</td>
</tr>
<tr>
<td>10</td>
<td>$\beta$</td>
<td>$-\text{S(}\text{CH}<em>2\text{)}</em>{10}\text{N}_2\text{NH}_2$</td>
<td>$-\text{H}$</td>
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<td></td>
</tr>
</tbody>
</table>

Hepta-6-guanidinealkyl $\beta$-cyclodextrin derivatives [104]

<table>
<thead>
<tr>
<th>Number</th>
<th>Cyclodextrin</th>
<th>$R_1$</th>
<th>$R_2$, $R_3$</th>
<th>Inhibition of Conductance $IC_{50}$, nM</th>
<th>Inhibition of Cytotoxicity $IC_{50}$, $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>$\beta$</td>
<td>$-\text{S(}\text{CH}_2\text{)}_2\text{NH}_2$</td>
<td>$-\text{H}$</td>
<td>$5.3 \pm 3.2$</td>
<td>$8.9 \pm 6.0$</td>
</tr>
<tr>
<td>12</td>
<td>$\beta$</td>
<td>$-\text{S(}\text{CH}_2\text{)}_3\text{NH}_2$</td>
<td>$-\text{H}$</td>
<td>$12.6 \pm 9.0$</td>
<td>$12.2 \pm 2.9$</td>
</tr>
<tr>
<td></td>
<td>Hepta-6-arylamine β-cyclodextrin derivative [104,105]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>β</td>
<td>–H</td>
<td>0.13 ± 0.10 0.8 ± 0.5</td>
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</tr>
<tr>
<td></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Cationic α- and γ cyclodextrin derivatives [105]</th>
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<tbody>
<tr>
<td>14</td>
<td>α</td>
</tr>
<tr>
<td>15</td>
<td>γ</td>
</tr>
<tr>
<td>16</td>
<td>α</td>
</tr>
<tr>
<td>17</td>
<td>γ</td>
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<table>
<thead>
<tr>
<th></th>
<th>Novel β-cyclodextrin derivatives [107]</th>
</tr>
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<tbody>
<tr>
<td>18</td>
<td>β</td>
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<tr>
<td></td>
<td><img src="image3.png" alt="Image" /></td>
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(Continued)
Table 38.2 Polyvalent cationic cyclodextrin blockers of PA 63 channel conductance (Continued)

<table>
<thead>
<tr>
<th>Number</th>
<th>Cyclodextrin</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;, R&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Inhibition of Conductance IC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
<th>Inhibition of Cytotoxicity IC&lt;sub&gt;50&lt;/sub&gt;, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>β</td>
<td>-H</td>
<td>n/a</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>β</td>
<td>-H</td>
<td>n/a</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>β</td>
<td>-H</td>
<td>n/a</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>β</td>
<td>-H</td>
<td>n/a</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>β</td>
<td>-H</td>
<td>n/a</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>Cyclodextrin</td>
<td>Inhibition of Conductance IC$_{50}$, nM</td>
<td>Inhibition of Cytotoxicity IC$_{50}$, μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>--------------</td>
<td>----------------------------------------</td>
<td>-----------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>β S NH$_2$ N H N H – H</td>
<td>n/a</td>
<td>26 ± 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>β S NH$_2$ N H N H – H</td>
<td>n/a</td>
<td>3.2 ± 1.9</td>
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<td></td>
</tr>
<tr>
<td>26</td>
<td>β S NH$_2$ N H N H – H</td>
<td>n/a</td>
<td>20 ± 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>β S NH$_2$ N H N H – H</td>
<td>n/a</td>
<td>&gt;100</td>
<td></td>
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<tr>
<td>28</td>
<td>β – H</td>
<td>n/a</td>
<td>4.1 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>β – H</td>
<td>n/a</td>
<td>2.1 ± 0.2</td>
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</table>
in vitro, in cell cultures, and in vivo (in the case of anthrax toxin) activity against four different binary bacterial toxins (i.e., anthrax, C2, iota, and CDT), thus representing the potential for the development of universal blockers against these toxins. The cyclodextrins, which are cyclic oligomers of glucose, have a long and successful history of being employed in the pharmaceutical, agrochemical, environmental, cosmetic, and food industries because of their ability to form water-soluble inclusion complexes with different small molecules and portions of large molecules. The cyclodextrins are not immunogenic and have shown low toxicity in animal and human studies. Moreover, because thousands of variations of the cyclodextrins with different ring sizes and random or specific chemical modifications are available, reliable methods for cyclodextrin synthesis and selective modifications have been developed.

The idea to use 7+βCD derivatives to inhibit anthrax toxins was based on the wealth of previous research. It was demonstrated that the heptameric pore of α-HL of S. aureus can be partially blocked by sevenfold symmetrical β-cyclodextrins. As discussed above, the positively charged tetraalkylammonium ions were reported to block ion current through PA63 channels apparently interacting with the negative charges on the channel walls and the ϕ-clamp. The internal diameter of the PA63 heptameric prepore was estimated to be 20–35 Å, with the PA63 pore’s constriction being as narrow as 12 Å. β-cyclodextrin molecules are naturally occurring cyclooligosaccharides, which contain seven α-(1,4)-D-glucopyranose subunits linked through α-(1,4) glucosidic bonds. The primary (C-6) and secondary (C-2 and C-3) OH-groups can be used as the potential sites for rational modifications. The OH-groups at positions 2 and 3 form hydrogen bonds and are required to keep the molecule rigid, whereas the OH-group at position 6 is a favorable site to introduce modifications.

These findings directed the rational design and custom synthesis of several 7+β-CD molecules, which have an external diameter of about 15 Å and carry seven positively charged amino groups covalently linked to a cyclodextrin core by hydrocarbon linkers. The authors identified several potent inhibitors of anthrax toxins showing that they act by completely inhibiting PA63 single-channel conductance following a two-state Markov model. To examine the effect of the nature of the linked positively charged ligands, a group of hepta-6-guanidine β-cyclodextrin derivatives was tested, in which positive charges were distributed between two nitrogens of the guanidine moiety. Activity of the guanidine-substituted cyclodextrin compounds was decreased only slightly compared to the aminoalkyl analogs. To identify an optimum length of the linkers which connect the positively charged amines to the cyclodextrin core, the blocker effect was examined using a group of hepta-6-thioaminoalkyl derivatives with alkyl spacers of various lengths both against PA63 channels in planar lipid bilayer membranes and in cell models.

From a combination of measurements on pore-blocking activity of 7+βCDs in planar lipid membranes and on inhibition of lethal toxin cytotoxicity in cell assays, it was shown that there is some optimal length of the alkyl spacers (between three and eight CH2-linkers) connecting positively charged amines to the cyclodextrin core. Shorter spacers were less effective in inhibition of the channels, apparently
Inhibitors of pore-forming toxins

because of size and mobility restrictions. Longer spacers exhibited higher toxicity to the RAW cells, and accordingly, induced instability of the bilayer lipid membranes [104]. The most significant effect in 7+βCDs activity was achieved when the nature of the spacers carrying amines was modified. Hepta-6-arylamine βCD derivatives, which, in addition to the functional amino-groups, have one phenyl group carried by each thio-hydrocarbon spacer, showed an enhanced binding affinity both in vitro and with cellular assays. One of the derivatives, per-6-S-(3-aminomethyl)benzylthio-β-cyclodextrin (AMBnTβCD) was subsequently chosen for more detailed investigation. In planar lipid bilayers, AMBnTβCD blocked PA63 with $K_D = 0.13 \pm 0.1$ nM and protected cultured macrophagelike cells from intoxication with anthrax lethal toxin (PA+LF) at $IC_{50} = 0.5 \pm 0.2$ μM [104,105]. We believe that these values are the best among the published data for the small-molecule PA63 pore blockers. Most important, AMBnTβCD completely protected Fisher F344 rats from intoxication with lethal toxin and, when administered in combination with the antibiotic ciprofloxacin, significantly increased the survival of mice in an infection model of anthrax [112]. These data show an emergent value of the 7 + βCD as a scaffold for design of polyvalent pore inhibitors. More generally, this finding once again demonstrates an advantage of the rational drug design over the traditional time- and money-consuming high-throughput screening of libraries of thousands of molecules, which often produces a hit rate lower than 1%. Indeed, from about 100 custom-synthesized 7 + βCDs, about two-thirds of the compounds were protective against the lethal toxin within cell assays, and nearly all compounds tested so far showed strong (nM) or moderate (μM-nM) channel blocking in planar lipid bilayers.

The importance of PA63 in targeting anthrax toxins has been questioned recently after the discovery that the anthrax toxin LF component remains active in cells and in animal tissues for days [113]. In particular, LF can be transported not only into the cytosol but also into the lumen of endosomal intraluminal vesicles. The vesicles fully protect LF from proteolytic degradation and significantly later fuse and release LF into the cytosol [114]. These findings may indicate that the focus of anthrax antitoxin research should be shifted toward LF. In our view, a successful anthrax therapy of the future might include combination polytherapies, where the action of the effective antimicrobials will be enhanced by the antitoxin agents specifically designed to target the A (LF, EF) and B (PA) components of the toxin. The various antitoxin agents must be also stockpiled to provide alternative options in the case of bacterial resistance or low response. It is also important to emphasize that, in contrast to any LF-targeting molecules, the PA63 channel blockers are expected to protect against both LF and EF. Moreover, the cationic β-cyclodextrin derivatives were also universally effective against related clostridial binary toxins (Figure 38.3). The most active of them so far identified is a β-CD-based inhibitor of PA63, AMBnTβCD. It fully protects cultured cells from intoxication with three clostridial binary toxins (C2, iota [106] and CDT [84]) by inhibiting the membrane translocation of the A components (C2I, Ia, CDTa) and blocking the ion current through heptameric pores formed by C2IIa and Ib in planar lipid bilayers in vitro [106]. Because PA63 insertion is always directional, trans-blocker addition was also examined [77]. Note that most of the experiments were performed under cis-side application of the blocker, which is believed to
correspond to a physiologically relevant endosome-facing cap-side of the channel. The trans addition of the 7+βCD resulted in an irreversible transition of the channel to a low-conductance substate. This finding indicates that the blocker can enter PA63 from either side, but it binds to different sets of amino acid residues and does not translocate through the pore because of the strong binding and size restrictions.

To examine the original idea about the importance of the blocker/pore symmetry complementarity, both sixfold symmetrical αCD carrying six positive charges and eightfold symmetrical γCD carrying eight positive charges were investigated. It was shown that even though the sevenfold symmetry of the blocker molecules complementing the heptameric structure of the channels was important, it was not a strict requirement for effective blockage. Thus, both 6+αCD and 8+γCD were able to block the PA63 channel in planar lipid membranes [105] with 6+αCD binding being significantly weaker and 8+γCD binding being comparable of that of 7+βCD. With that, activity of the 8+γCDs tested with cell assays was equal to or slightly increased compared with the 7+βCDs; activity of 6+αCD was not strong enough to make the

---

**Figure 38.3** Blocking bacterial toxins at the single-channel level. (A) Conductance of PA63, C2Ia, and Ib channels in the absence (top) and presence (middle row) of AmPrbCD (Table 3, #3), and AMBnTbCD (bottom row) (Table 3, #13) blockers. AMBnTbCD, which has phenyl groups carried by each thio-hydrocarbon spacer, displays a significantly longer binding lifetime compared with AmPrbCD, which lacks the phenyl groups. The time between the blockage events, characterizing the on-rate of the binding reaction, seems to be practically unchanged. (B) The PA63 F427A mutant shows much shorter blockages for both AmPrbCD (middle track) and AMBnTbCD (bottom track) blockers. Recordings shown at 1-ms time resolutions were taken in 1 M of KCl at pH 6 and 50 mV applied voltage. Reprinted with permission from [115]. Copyright 2012 Biophysical Society.
sixfold symmetrical blockers protective against the anthrax toxin. One can argue that the preserved activity of the 8+γCDs is related to the observation of PA$_{63}$ octameric pores [31]. However, both 7+βCDs and 8+γCDs were able to block any particular single PA$_{63}$ channel examined in the planar bilayers.

These findings show that the development of new pore-inhibiting molecules should not rely exclusively on the matching of blocker/pore symmetries and electrostatic interactions. The cyclodextrin molecules apparently serve as ring-shaped platforms that carry multiple positive charges and aromatic groups toward their pore targets. Inside the pore, these residues interact with hydrophobic and negatively charged groups provided by the seven subunits of the channels. The less active 6+αCD can contribute only six ligands to the blocker-pore-binding reaction and, with its smaller 13.7-Å external diameter, it may not be large enough to allow for a proper fit in the pore’s lumen. 8+γCD, which shows similar activity to 7+βCDs, can deliver an additional positively charged ligand. This extra charge group can likely compensate for the increased size of the 8+γCD blocker, which may limit its flexibility inside the channel. More recently, several novel groups of cationic βCD blockers, which differed in number, arrangement, and face location of the positively charged substituents were synthesized and tested in cell assays against the anthrax toxin [107]. Once again, the measurements confirmed that the cationic cyclodextrins designed based on the symmetry complementarity model alone could not provide an increased binding affinity toward the PA$_{63}$ channel [107]. Moreover, introduction of seven additional positive charges to yield 14+βCD did not increase the compound’s activity but significantly increased its cytotoxicity [107].

Based on single-channel, single-molecule measurements made under different salt, voltage, and temperature conditions [115,116], the nature of the physical forces involved in the 7+βCD/pore binding reaction was analyzed. At moderate and low salt concentrations, the analysis revealed the contribution of long-range Coulomb interactions, whereas at high salt concentrations, the salt-concentration-independent short-range interactions was shown to predominate. At KCl concentrations lower than 0.5 M, the 7+βCD activity increases in the order Ib < C2IIa < PA$_{63}$, as does the cationic selectivity of the channels. The difference in the affinity of small-molecule cationic blockers toward the PA$_{63}$, C2IIa, and Ib channels has been explained by a different number of the negatively charged amino acids on the lumen of these pores [72,74,83]. Therefore, the observed pattern for the 7+βCD binding may indicate that the positive charges of the βCD blockers interact with the negatively charged amino acids in the channel lumen. At the same time, binding of the most effective AMBnTβCD blocker is further enhanced by the presence of the aromatic groups, which presumably interact with certain conserved residues (most likely with the ϕ-clamp [38]) in the lumen of the PA$_{63}$, C2IIa, and Ib channels. As an alternative, the presence of aromatic groups could change the conformation of the linkers carrying the positively charged groups, which could create additional favorable conformations of the blocker molecules, thus stabilizing their binding to the target. To summarize, a number of Coulomb and salt-concentration-independent short-range interactions that act simultaneously within a single binding pocket of the pore lumen are involved. The particular origin of these forces will require further experimental and theoretical efforts because quantitative interpretation of blocker/pore interactions might be complicated by multiple factors.
Among these factors are the hydration states of the blocker molecule both in the bulk and inside the pore lumen [117], determined by a sophisticated interplay between the hydrophobic effects and different electrostatic components, and the ambiguity regarding possible interaction-induced changes in the blocker and channel structures. Currently, we can assume that in addition to hydrophobic interactions, many others, such as aromatic-aromatic, $\pi$-$\pi$, cation-$\pi$ interactions, hydrogen bonding, and van der Waals interactions, might be involved in the cyclodextrin/pore binding. There is the hope that careful Monte Carlo, molecular dynamics simulations, and multiscale modeling, combined with the existing and future experimental data, will shed light on the relative contribution from the different types of physical forces. Note that this analysis refers only to the reversible ion current blockages, which were shown to obey the two-state Markov process. The designed 7+βCD compounds also act by enhancing voltage gating of the PA$_{63}$, C2IIa, and Ib channels, making their closed state more favorable [77]. Because the very nature of the voltage gating in β-barrel channels remains unclear, it is hard to estimate to what extent this second type of 7+βCD action observed in vitro could influence the blocker activity in vivo.

The pore-blocking activity of the cationic polyvalent compounds was also recently explored using another group of potential antitoxins called PAMAM dendrimers. These dendrimers are repeatedly branched polymers, with all bonds emanating from a central core [118] (Table 38.3). In particular, the commercially available PAMAM-

<table>
<thead>
<tr>
<th>Number</th>
<th>PAMAM dendrimer generation</th>
<th>Measured diameter (Å)</th>
<th>NH$_2$ surface group number</th>
<th>Inhibition of conductance IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>15</td>
<td>4</td>
<td>128 ± 44 nM</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>22</td>
<td>8</td>
<td>5.3 ± 2.6 nM</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>29</td>
<td>16</td>
<td>7.15 ± 4.7 nM</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>36</td>
<td>32</td>
<td>5.0 ± 1.4 nM</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>45</td>
<td>64</td>
<td>2.4 ± 1.3 nM</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>97</td>
<td>1024</td>
<td>0.22 ± 0.08 nM</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>135</td>
<td>4096</td>
<td>0.16 ± 0.07 nM</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>15</td>
<td>4</td>
<td>16.5 ± 3.3 μM</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>22</td>
<td>8</td>
<td>4.6 ± 1.7 μM</td>
</tr>
<tr>
<td>10</td>
<td>0 dendrons</td>
<td></td>
<td>2</td>
<td>26 ± 7 nM</td>
</tr>
<tr>
<td>11</td>
<td>1 dendron</td>
<td></td>
<td>4</td>
<td>4.9 ± 0.7 nM</td>
</tr>
<tr>
<td>12</td>
<td>2 dendrons</td>
<td></td>
<td>8</td>
<td>4.2 ± 0.9 nM</td>
</tr>
</tbody>
</table>
dendrimers are based on an ethylene diamine core and an amidoamine repeat branching structure and are available as regularly branched, highly monodisperse starburst polymers of different generations (G0–G10) varying in size ($d = 15–135\text{Å}$) and surface charge ($z = 4$ to $4096$). According to the dendrimer manufacturer, Dendritech Inc. (based in Midland, MI), each consecutive growth step represent a new “generation” of dendrimer with a larger molecular diameter and twice the number of reactive surface sites. Unlike the conventional linear polymers, dendrimers can be regulated by well-controlled branched chemical syntheses [119], resulting in the polymers possessing unique characteristics of nanosize range, monodispersity, rigid and stable globular polyvalent structure, and highly regulated number of functional groups and surface charges [119]. Various emergent industrial and medical applications of the dendrimers include light- and energy-harvesting, catalyst and optoelectronic applications, drug and gene delivery, manufacture of artificial bones, biomedical coating, and usage as antibacterial, antiviral, and antiparasitic agents [120–124]. Using a combination of planar lipid bilayer and cell-based experiments, the PAMAM dendrimers were shown to block ion currents through PA$_{63}$ and C2IIa pores and also inhibit channel-facilitated transport of the enzymatic components [118]. Interestingly, the in vitro IC$_{50}$ values of the PAMAM/PA$_{63}$ binding reaction (0.16–230 nM, depending on generation) were comparable to that of the very first rationally designed PA$_{63}$ β-CD based inhibitor, AmPrβCD (0.55 nM) [77]. This blocker was selected out of dozens of β-CD based molecules [104]. Further optimization would clearly increase the pore-binding affinity of these polyvalent molecules. In the bilayer lipid membranes, all tested dendrimers inhibited PA$_{63}$ and C2IIa conductance in a concentration-dependent manner, with generations G1, G2, and G3 showing stronger binding affinity when compared with the low-generation G0 and high-generation G4, G8, and G10 PAMAM dendrimers. G1, G2, and G3 dendrimers carry, respectively, 8, 16, and 32 surface primary amines and have the measured diameter of 22, 29, and 36 Å, which indicates that these blockers are small enough to enter the channels from the cis (cap-side) compartment. Indeed, the trans (stem) side addition of the G0-NH$_2$ and G1-NH$_2$ PAMAM dendrimers led to about 130 and 870 times weaker binding, respectively, compared with the cis-side addition under the constant cis-side positive 20-mV transmembrane voltage. The study also reports that the PAMAM dendrimers of generation 2 and higher exhibited effects on the morphology of the tested cells on their own, decreasing the number of viable cells. This finding is not surprising considering the number of surface charges (16–4096) these polymers hold. However, the study examines several possible solutions for the blocker’s toxicity problem. In particular, it was earlier demonstrated that an increase in activity, and therefore, a more favorable therapeutic window, is achieved with the molecules that were partially degraded to the so-called fractured or imperfect dendrimers [125]. Likewise, the authors report that pore-blocking activity of G1-NH$_2$ dendrons carrying four surface primary amines was about 26 times higher (IC$_{50}$ = 4.9 nM) than that of the four positively charged G0 dendrimer (IC$_{50}$ = 128 nM). The observed effect could be explained by an increase in mobility of the surface primary amines carried by the G1 dendron, which have higher flexibility for finding the binding sites in pore lumen, better access of the tertiary amino groups to these sites, or both.
**Targeting membrane-perforating bacterial toxins**

Despite the simplicity of the idea, the number of molecules under development that specifically target membrane-perforating toxins, which is the largest group of bacterial toxins, is relatively low. However, the *in vitro* biophysical studies on these toxins are simplified by the fact that the biological action of the membrane-perforating bacterial toxins is based on the permeabilization of the host cell’s membranes. The multistage and often not-well-understood mechanism of the uptake of enzymatic components of the AB-type toxins does not apply to PFTs. Therefore, disabling the PFTs could simply imply a physical obstruction of the virulent ion conductive channels formed by these toxins. Moreover, several membrane-perforating PFTs (for instance, α-HL of *S. aureus* and aerolysin of *Aeromonas hydrophila*), were extensively studied using the model bilayer lipid techniques in a wide variety of biosensing applications (reviewed in [126]). Because the basic principles of the biosensing nanopore biotechnology could be extended to the pore blocking inhibitor design, we hope that a number of new interesting discoveries would soon follow this backlog. Several studies where the molecules specifically targeting the membrane-perforating bacterial toxins were designed and tested are discussed in the next section.

**Inhibiting staphylococcal membrane-perforating toxins**

*S. aureus* is one of the most frequently isolated bacterial strains in routine clinical laboratory hospital testing [127,128]. Recently, rapt attention has been given to the toxins secreted by *S. aureus* because of the widespread, highly virulent [129], and multidrug-resistant type of the bacterium, the so-called methicillin-resistant *S. aureus*, or MRSA [130,131]. Among numerous virulence factors, *S. aureus* produces several PFTs, including α-HL, γ-hemolysin, and leukocidins. Different aspects of the *S. aureus*’s membrane-perforating toxin action are discussed in Chapter 25 of this book.

The α-HL is secreted as a water-soluble, 293-amino-acid monomeric polypeptide with a molecular mass of 33kDa. It binds to a target cell membrane, oligomerizes, and then forms heptameric complexes on the cell surface [132–137]. There are several reports of formation of hexameric α-HL complexes [138–140]. In the pioneering electrophysiological studies by Krasilnikov and co-authors more than 30 years ago, the oligomeric α-HL complexes were successfully incorporated into the model lipid bilayer membranes, where they formed large, slightly anion-selective, water-filled pores of about 1nS conductance (in 1M of KCl at room temperature) [138,141]. The 1.9-Å-resolution crystal structure of a heptameric α-HL [134] shows a hollow, 100 Å × 100 Å heptamer, which has a mushroom-like shape consisting of the stem, cap, and rim domains. The cap has a diameter of about 100 Å [127] and the internal diameter of the α-HL channel ranges from about 6 to approximately 50 Å. Two constriction zones with radii of 9 Å and 6–7 Å were detected in the channel lumen, with the larger one being located closer to the cis side [142]. The α-HL channel stability, dimensions, and structural robustness determine the wide usage of this bacterial toxin in a variety of biotechnological applications, which are not necessarily directly related to the toxicity of this protein.
Large, open β-barrel channels, such as α-HL, are the ideal candidates for the stochastic resistive-pulse sensing of different types of molecules [143], ranging from small molecules to polymers. One of the fascinating examples of α-HL’s sensing properties is its ability to integrate the β-cyclodextrin (βCD) molecules, first reported by Bayley and colleagues [110]. The authors equipped a single α-HL pore with the sevenfold symmetrical βCD-based adapters, which could noncovalently bind to the pore lumen and mediate channel blocking by the different organic analytes, such as adamantaneamine hydrochloride, adamantane carboxylic acid, promethazine, and imipramine. Later, the cyclodextrin’s ability to enter a protein pore and reversibly obstruct it was explored in a rational design of α-HL blockers [144,145] (Table 38.4). The authors advanced the idea of enhancing the blocking ability of the channel-blocking inhibitors using compounds of the same symmetry as the target pore. One of the newly designed hepta-6-substituted β-cyclodextrin derivatives, named IB201, not only blocked ion conductance through the channels formed by α-HL in planar bilayers [144], but also was able to protect rabbit red blood cells from α-HL-induced hemolysis in low micromolar concentrations [144]. IB201 prevented α-HL-mediated alveolar epithelial cell lysis and mortality associated with S. aureus pneumonia in a murine model of infection [145]. Using single-channel measurements in the bilayer lipid membranes, the authors showed that in contrast to 7+βCD binding to PA63, the α-HL interaction with IB201 was irreversible, at least within the time period of the experiment. An IB201 cis-addition induced α-HL to switch to a low conductive substate, with the residual conductance of the closed state ranging between 1% and 15% of the open channel conductance. The effect was stronger when trans-negative voltages were applied, showing that the introduction of positive charges to the

<table>
<thead>
<tr>
<th>Number</th>
<th>Cyclodextrin</th>
<th>R_1</th>
<th>Inhibition of conductance IC_{50} nM</th>
<th>Inhibition of cytotoxicity IC_{50} μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α</td>
<td></td>
<td>&gt;5000</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2</td>
<td>β</td>
<td></td>
<td>About 50</td>
<td>3.3 ± 2.3</td>
</tr>
<tr>
<td>3</td>
<td>γ</td>
<td></td>
<td>&gt;5000</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>
cycloexetirin molecule led to its ability to block this anion-selective channel from the physiologically relevant cis side. Importantly, in contrast to the PA₆₃ blockage, α-HL was selectively blocked only by the sevenfold symmetrical β-CDs, whereas sixfold αCDs and eightfold symmetrical γCDs carrying the same substituent groups were not effective with cell assays and caused no αCD ion current decrease in the planar lipid membranes [105]. At the same time, because only a very small number of compounds from the available library of 7+βCD derivatives showed α-HL inhibition similar to the IB201 action, it is unclear at the moment what particular molecular interactions are involved in the α-HL blocking.

Apart from α-HL, a family of staphylococcal pore-forming cytolysins, γ-hemolysin (Hlg), leukocidin (Luk), and Panton-Valentine leukocidin (PVL), are produced as a result of the interaction of two distinct polypeptides: the so-called class F and class S components [146]. The 2.5-Å-resolution crystal structure of a β-barrel transmembrane protein γ-hemolysin shows an octameric bicomponent pore structure [147]. The model bilayer measurements conducted on another bicomponent pore-forming octameric leukocidin, Luk, revealed a conductance of 2.5 nS in 1 M of KCl, which is about 2.5-fold higher than that of α-HL [148–150]. Recently, the staphylococcal bicomponent pore-forming leukotoxins were inhibited by selected p-sulfonato-calix[n]arenes (SCns) [151]. The authors explored structural characteristics and stoichiometry of the S. aureus toxins and selected various potential pore-blocking compounds of different hydrodynamic radii, shapes, symmetries, and charges, focusing on the molecules that would be potentially compatible with the leukotoxin’s pore lumens. The series of the tested compounds included several cyclic compounds, cyclodextrins, and several newly designed cyclic peptides [c(GF)₃, c(GF)₄, and c(KD)₃]. Among the compounds probed, calixarenes SC₆ and SC₈ demonstrated explicit inhibitory effects when tested on red blood cells (RBCs) and liposomes. Remarkably, even though the authors initially chose their potential inhibitors based on the intention to directly obstruct the upper ring of leukotoxin pores, the mechanism appears to be different. Thus, at −100 mV transmembrane voltage, only 25% and 35% of current reduction was observed in the presence of 50 μM of SC₆ and SC₈, respectively. At the same time, the interaction of SCn with leukotoxins turned out to be specific for class S rather than class F protein-composing leukotoxins. SCns are believed to interact with class S proteins via electrostatic rather than purely steric interactions, and to specifically prevent the binding of class S proteins to membranes, inhibiting pore formation. Importantly, the concentrations of SCn that are toxic for cells were fourfold to fivefold greater than their inhibitory concentrations. Besides, the activity of SC₈ was also tested against PVL action in a rabbit noninfectious model, where SC₈ was able to considerably reduce the inflammation induced by 600 ng of PVL in rabbit eyes.

**Inhibiting ETX of C. perfringens**

ETX, the major virulence factor produced by the B and D types of C. perfringens [152], is another potential critical target for the development of channel-blocking
pharmaceutical agents. In recent years, several excellent reviews discussing many different aspects of the ETX’s action have been published [153–156]. The activated ETX represents one of the most potent bacterial toxins after botulinum and tetanus neurotoxins [157]. The toxin is responsible for a rapidly fatal enterotoxemia in herbivores when their gastrointestinal tracts are colonized by this bacterium, leading to in situ toxin production [20,158,159]. An estimated 7-μg intravenous lethal human dose was reported [158], resulting in ETX being classified as a CDC category B agent. An ETX monomer [160] consists of three structural domains. The N-terminal domain I is responsible for the receptor binding; domain II is thought to be involved in oligomer and pore formation, and the C-terminal domain III is believed to mediate ETX membrane insertion. ETX is produced in a low-active prototoxin form [161], which has to be activated into a highly potent toxin by the proteolytic removal of 11 or 13 N-terminal and 29 C-terminal amino acid residues [162]. ETX later oligomerizes in the synaptosomal membrane within the detergent-insoluble microdomains (so-called lipid rafts) of Madin-Darby canine kidney (MDCK) cells, forming a heptameric prepore on the membrane surface [163]. The prepore later transforms into an aerolysin-like [160] β-barrel heptameric transmembrane channel. The ETX pores are able to increase cell permeability to small molecules and ions [20,167–169] and rapidly depolarize membranes [163].

In the bilayer lipid membranes, no receptors are required for the ETX channel reconstitution [170,171]. ETX forms wide, slightly anion-selective general diffusion pores with a single-channel conductance in the range of 440–640 pS in 1 M of KCl [170,171]. The channel was suggested to be permeable to solutes up to a molecular mass of at least 1 kDa [170]. Without the solved crystal structure of the ETX heptamer, polymer partitioning experiments provide some insight into the ETX’s pore functional shape and size [172]. Polyethylene glycol (PEG) partitioning was established to be highly asymmetrical; the trans opening of the ETX pore allowed for penetration of much larger polymer molecules compared to its cis opening. These measurements may indicate an asymmetrical (e.g., conical) shape of the ETX pore, with tentative radii of the openings of 0.4 nm and 1.0 nm on the cis and trans sides, respectively. To analyze the charge distribution along the ETX pore, the ionic selectivity of the ETX pore was investigated by measuring reversal potentials in the oppositely directed gradients of potassium chloride aqueous solutions [172]. Remarkably, the asymmetry in the reversal potential under the KCl concentration gradient was reported to be the opposite of one for the conical nanopores with a uniformly spread surface charge [173,174]. Thus, the selectivity was “salted out” more easily from the wide trans-opening of the ETX channel. This may suggest that the amino acid residues that carry the positive charge responsible for the anionic selectivity of the ETX pore [170,171] are shifted toward the trans-side of the channel instead of being localized at its narrow cis opening. The only study searching for ETX channel inhibitors used a cell-based, high-throughput screening assay to screen a library of 151,616 compounds for their ability to inhibit ETX cytotoxicity [175]. The authors identified three compounds inhibiting ETX: N-cycloalkylbenzamide, furo[2,3–b]quinoline, and 6H-anthra[1,9–cd]isoxazol. Because none of these three compounds inhibited ETX cell binding or oligomerization, along with other possible mechanisms, they might have acted by blocking the ETX pores.
Comparison with pore-blocking inhibitors of ion-selective channels of neurophysiology

In contrast to the relatively newly emergent field of pore-blocking toxin inhibitors, ion-selective channels of neurophysiology represent the most extensively studied membrane proteins in functional, structural, and pharmacological aspects [176]. The ion-selective channels of excitable cells are vital for information transfer by electrical signals between the various cells in different parts of the body. In vertebrates, these channels coordinate the functions of many organs—namely, allowing the processing of information in the brain, environmental sensing, and the delivery of signals from the brain to peripheral organs, including the control of muscle contractions and the release of hormones. It comes as no surprise that advances in the structure-inspired design of new drugs that modulate properties of the ion-selective channels are quite extensive [177–188]. Importantly, many described ion channel modulators are not only used as versatile research tools, but also have successfully found their way into pharmacology for treating a broad spectrum of diseases such as epilepsy, chronic pain, cardiac arrhythmia, ischemia, memory disorders, hypertension, type 2 diabetes, and many others.

It is worth comparing the affinity of the PFT blockers described in the previous sections with a wealth of data on the blockage of ion-selective channels of neurophysiology. Within the limits of this chapter, we have been primarily interested in the comparison of the small-molecule nonpeptide channel blockers. The full list of available inhibitory constants for blockers of potassium, sodium, and calcium channels is lengthy, even if it is restricted to nonpeptide ion channel inhibitors. Table 38.5 gives only a few representative examples of the most efficient blockers of the ion channels of neurophysiology. Remarkably, the IC₅₀ values for the small-molecule blockers of

Table 38.5 Examples of the most potent nonpeptide blockers of ion-selective channels of neurophysiology

<table>
<thead>
<tr>
<th>Number</th>
<th>Channel</th>
<th>Blocker</th>
<th>IC₅₀ (nM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Potassium, Kᵥ4.3</td>
<td>Nicotine</td>
<td>34</td>
<td>[189]</td>
</tr>
<tr>
<td>2</td>
<td>Potassium, Kᵥ11.1</td>
<td>Astemizole</td>
<td>48</td>
<td>[190]</td>
</tr>
<tr>
<td>3</td>
<td>Potassium, Kᵥ11.3</td>
<td>Sertindole</td>
<td>43</td>
<td>[191]</td>
</tr>
<tr>
<td>4</td>
<td>Sodium, Naᵥ1.1</td>
<td>Tetrodotoxin</td>
<td>6</td>
<td>[180,192]</td>
</tr>
<tr>
<td>5</td>
<td>Sodium, Naᵥ1.6</td>
<td>Tetrodotoxin</td>
<td>1</td>
<td>[180,193]</td>
</tr>
<tr>
<td>6</td>
<td>Calcium, Caᵥ1.2</td>
<td>Devapamil</td>
<td>50</td>
<td>[181,194]</td>
</tr>
<tr>
<td>7</td>
<td>Inwardly rectifying potassium, Kᵢ2.1</td>
<td>Spermine</td>
<td>0.9</td>
<td>[187,195]</td>
</tr>
<tr>
<td>8</td>
<td>Calcium-activated potassium, Kᵥc1.1</td>
<td>Paxilline</td>
<td>1.9</td>
<td>[185,196]</td>
</tr>
<tr>
<td>9</td>
<td>Calcium-activated potassium, Kᵥc2.1</td>
<td>UCL1684</td>
<td>0.8</td>
<td>[185,197]</td>
</tr>
<tr>
<td>10</td>
<td>Calcium-activated potassium, Kᵥc2.2</td>
<td>UCL1684</td>
<td>0.28</td>
<td>[185,198]</td>
</tr>
</tbody>
</table>
Inhibitors of pore-forming toxins

Ion-selective channels of neurophysiology are nearly an order of magnitude higher than that for AMBnTβCD inhibition of current through the anthrax PA63 channel (Table 38.2), characterized by IC₅₀ = 0.13 ± 0.10 nM. UCL1684 blocker, which inhibits the calcium-activated potassium channel KC2.2 with about the same affinity as AMBnTβCD, is the only exception.

**Concluding remarks**

Promising new developments in the rational design of pharmaceutical agents continue to occur. The structure-inspired drug design has allowed for development of numerous small-molecule blockers of the classical ion-selective channels of neurophysiology. The existing and emerging structural data on potassium, sodium, chloride, and ligand-gated channels have proved to be critically important in the de novo design and virtual screening of molecular libraries. Several viroporins, including the tetrameric proton-selective M2 channel from the viral envelope of influenza A virus, have also been traditionally considered as important targets for the development of pore-blocking agents. More recently, the efforts to rationally design pore-blocking compounds of immediate pharmaceutical interest were extended to include the so-called pores of virulence—the ion-conductive complexes formed by the pore-forming bacterial toxins or by their components. These novel compounds range from small-molecule blockers to polyvalent agents that match the symmetry of these pores, complement their internal structure in size, structure, charge, and hydrophobicity.

Remarkably, in vitro potency of the most active rationally designed antitoxins compares well with that of the ion-selective channels of excitable cells. However, there is a fundamental difference that medicinal chemists working toward developing effective blockers of toxin pores should keep in mind. One of the main challenges of designing modulators of ion-selective channels of neurophysiology is to find a compound that inhibits only one type of channels with high specificity. In contrast, the pore-blocking antitoxins are often expected to be universal. This chapter has provided a vivid example of one of the first successful designs of such wide-spectrum antitoxins. Indeed, the custom-synthesized, positively charged polyvalent cyclodextrin-based AMBnTβCD compound was shown to be protective against the cytotoxicity induced by four different toxins: anthrax toxins of B. anthracis [104], clostridial C2, iota [106], and CDT toxins [84]. The universality idea is easy to perceive and appreciate because a potent, wide-spectrum antidote would be expected to be active against more than one virulent agent. Searching for such a compound, we rely on the knowledge of the structure and function of molecular targets, and focus on the shared mechanisms that could be directionally targeted. In the case of β-CD-based antitoxins, the selection of inhibitors was based on exploiting the universal target common to these toxins—the heptameric cation-selective pores formed by the binding components of the binary bacterial toxins. By showing that blocker activity is determined by the sophisticated interplay between the different hydrophobic and electrostatic effects [115], researchers have opened ample avenues for rational discovery of the universal pore-inhibiting agents of the future. Thus, several synthesized azolopyridinium salts [97,98] and commercially
available cationic PAMAM dendrimers [118] were recently shown to be universally effective against both anthrax and C2 bacterial toxins. When optimized, these or other positively charged molecules might be considered for further pharmaceutical development. As with any drug design program, this path is not free of challenges; the ideal universal antidote is expected to be harmless against mammalian channels, and generally, to possess low toxicity. Even though the designed β-CD-based antitoxins showed relatively low toxicity in cell assays, positively charged compounds are often seen as less biocompatible than their neutral and negatively charged analogs. To remedy the potential cytotoxicity problem, numerous modern lead optimization methods suggest novel surface engineering approaches, allowing for masking the positive charges by a partial surface derivatization with chemically inert groups such as PEG or fatty acids. Alternatively, the compounds could be encapsulated into liposomes or micelles. For instance, poly(ethylene glycol)-b-poly(aspartic acid) micelles, while being stable at physiological conditions, can disintegrate in the endosomal acidic environment [199,200]. This feature would allow direct delivery of the blockers to the binary toxin targets. These discoveries lead one to hope that the inhibitive action of the rationally designed universal antidotes will be mostly limited to their intended targets, paving the way for the eventual use of these drugs in clinic.

Acknowledgments

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