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## Inhibition of Sodium Ion Channel Function with Truncated Forms of Batrachotoxin

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### Abstract

A novel family of small molecule inhibitors of voltage-gated sodium channels (Na<sub>v</sub>s) based on the structure of batrachotoxin (BTX) – a well-known channel agonist – is described. Protein mutagenesis and electrophysiology experiments reveal the binding site as the inner pore region of the channel, analogous to BTX, alkaloid toxins, and local anesthetics. Homology modeling of the eukaryotic channel based on recent crystallographic analyses of bacterial Na<sub>v</sub>s suggests a mechanism of action for ion conduction block.

### Graphical Abstract

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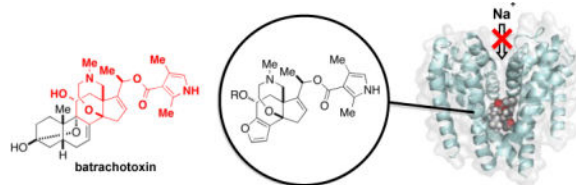
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Supplementary figures (S1–S6), experimental details and homology models are available in the supporting online material. Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.



Voltage-gated sodium ion channels ( $\text{Na}_V$ s) are responsible for the initiation and propagation of action potentials in excitable membranes.<sup>[1]</sup> Eukaryotic  $\text{Na}_V$ s are comprised of an  $\alpha$ -subunit, which assembles as four homologous domains (D1–D4) to form the central ion conduction pore, and two auxiliary  $\beta$ -subunits. Transient changes in membrane voltage potential effect rapid alterations in channel conformation, which are critical for proper ion gating. Biophysical and computational studies to characterize  $\text{Na}_V$  dynamics have been aided through structural investigations that include protein X-ray crystallography,<sup>[2]</sup> mutagenesis experiments,<sup>[3]</sup> and ligand binding studies.<sup>[4]</sup> The value of small molecules for interrogating  $\text{Na}_V$  structure-function is considerable, as SAR data in combination with site-directed mutagenesis have shaped current views of the molecular form of the eukaryotic channel structure.<sup>[5]</sup> These insights notwithstanding, the ability of small molecule  $\text{Na}_V$  modulators to alter ion gating kinetics by influencing conformational states of the channel is poorly understood.<sup>[6]</sup> Given the intensive interest in  $\text{Na}_V$ s as targets for drug design,<sup>[7]</sup> deeper insight into the complex molecular interactions between ligand and channel is sought.

Batrachotoxin (BTX), a natural product isolated from poison dart frogs of the genus *Phylllobates*,<sup>[8]</sup> is a potent modulator of  $\text{Na}_V$ s.<sup>[9]</sup> Binding of this molecule to the inner pore of the channel (Site II) elicits a multitude of functional responses, including a hyperpolarized shift on the voltage-dependence of activation, inhibition of both fast and slow inactivation, and reduction in ion selectivity.<sup>[10]</sup> The uniqueness of BTX as a  $\text{Na}_V$  agonist has motivated our efforts to understand the molecular details of its binding interactions with the channel and its structure-function properties. As part of this program, we have prepared simplified BTX-like structures **1** (Figure 1).<sup>[11]</sup> Electrophysiology recordings with wild-type and mutant  $\text{Na}_V$  isoforms demonstrate that BTX analogues comprising the C, D, and E rings of the natural product *block*  $\text{Na}_V$  current – a stark contrast to the behavior of BTX itself. Protein mutagenesis data suggest that BTX analogues lodge in the inner pore lining of the channel, thus sharing a common receptor site with the parent compound. Surprisingly, both enantiomers of **1** display nearly identical potency as  $\text{Na}_V$  inhibitors.<sup>[12]</sup> A homology model of the binding interactions of **1** with the channel pore is presented in an attempt to rationalize the lack of stereorecognition in the receptor site. Collectively, these results establish **1** as a novel chemotype for  $\text{Na}_V$  inhibition and as a chemical probe for understanding the molecular mechanism of  $\text{Na}_V$  function.

Early structure-activity relationship studies on the effects of semi-synthetic BTX derivatives against  $\text{Na}_V$ s highlight the importance of the ester and amine groups in addition to oxygen functional groups at C3, C9, and C11 for toxin function.<sup>[13]</sup> Protein mutagenesis experiments,<sup>[3]</sup> ligand binding studies,<sup>[4]</sup> and competitive ligand displacement assays<sup>[14]</sup> give substantive evidence that toxin interaction with  $\text{Na}_V$  is localized to Site II. Homology

modeling and single-point mutagenesis data suggest that the C/D/E ring portion of BTX makes primary contacts with the inner pore lining.<sup>[3f,15]</sup> The lack of availability of BTX from natural sources restricts access to new, modified BTX compounds and further examination of this model.<sup>[9c]</sup> Investigations of structure-function with BTX analogues and protein mutants can give insight into the unique ability of BTX to influence voltage activation and inactivation through binding the central cavity of the channel. Accordingly, *de novo* chemical synthesis of the toxin and related structures is the only current available strategy for accessing such probes.<sup>[16]</sup>

Truncated forms of BTX comprised of the C/D/E ring units and C20 ester moiety are available in two steps from silyl ether **2** (Scheme 1). Derivatives that contain either the acylpyrrole group found in BTX or those bearing benzoate, naphthoate, cyclohexylcarboxylate, and butyrate are generated in good yields (33–95%) using either acid chloride or mixed-anhydride reaction partners. Coupling of a mixed-anhydride form<sup>[17]</sup> of BODIPY@ FL to **3** gives a fluorescent conjugate **1f**, which was prepared to examine the steric dimensions of the receptor site and for its potential as an imaging tool for ligand displacement assays.<sup>[14]</sup> Each of these compounds has been synthesized from **2** in multi-milligram quantities.

Electrophysiology measurements were performed in a whole-cell voltage-clamp format against the  $\alpha$ -subunit of the rat skeletal muscle Na<sub>v</sub> (rNa<sub>v</sub>1.4), heterologously expressed in Chinese hamster ovarian (CHO) cells. Currents were elicited by 10-ms step depolarizations from a holding potential of –100 to 0 mV and were recorded at steady state. In this assay, a channel modified by BTX, which binds preferentially to the open-state of the channel (use-dependence) exhibits a sustained current.<sup>[3,10]</sup> By contrast, application of benzoate **1a** or naphthoate **1b** (100  $\mu$ M) shows partial *block* of Na<sup>+</sup> current in a use-dependent manner (Figure 2A, data shown for **1b**). Measured IC<sub>50</sub> values for these two compounds are 81.4  $\pm$  4.8 and 17.4  $\pm$  0.4  $\mu$ M, respectively (Figures 2B, S1). More surprisingly, experiments performed with either antipode of **1b** (i.e., **1b\***) demonstrate that inhibition of the channel by these agents is not stereoselective.

Other ester derivatives display similar inhibitory activities to **1a** and **1b** with the most potent having an IC<sub>50</sub> value as low as 11.1  $\pm$  0.3  $\mu$ M (**1c**). Notably, block of Na<sup>+</sup> current is also observed with the sterically large BODIPY@ FL conjugate **1f** (19.2  $\pm$  2.4  $\mu$ M). Assuming **1f** shares a common receptor with BTX and other BTX analogues, this result suggests that a large open space may comprise the ligand binding site (*vide infra*). As with BTX, the absence of the ester group dramatically alters the affinity for channel binding.<sup>[13,18]</sup> Electrophysiology recordings with alcohol **3** show < 5% block at 1 mM concentrations. Use-dependent block of sodium ion conduction by **1a–1f** markedly contrasts the activity of BTX and establishes these molecules as a new class of Na<sub>v</sub> inhibitors.

Subsequent investigations of BTX C/D/E analogues were performed with naphthoate **1b** given the potency and ease of synthesis of this particular compound. Electrophysiology recordings with different Na<sub>v</sub> isoforms, which included rNa<sub>v</sub>1.2, hNa<sub>v</sub>1.5, and hNa<sub>v</sub>1.7, give measured IC<sub>50</sub> values that vary between 8–30  $\mu$ M (Figures 3, S2). The similar potency

of **1b** against channel subtypes is perhaps unsurprising in light of the nearly identical primary structure of the amino acids that are believed to comprise the inner pore.

Binding of BTX to  $\text{Na}_V$  hyperpolarizes the voltage-dependence of channel activation by  $-45$  mV, allowing a greater population of channels to open at resting membrane potentials.<sup>[3,9,10]</sup> In addition, BTX is known to inhibit fast inactivation. By contrast, electrophysiology experiments to test the influence of **1b** on channel activation and inactivation reveal that this derivative has only a slight influence on the steady-state voltage dependence of activation and inactivation (Figure 4). Binding of **1b** ( $25 \mu\text{M}$ ) to  $\text{rNa}_V1.4$  shifts  $V_{0.5}$  of activation by  $+4.9$  mV and the slope factor is increased by  $+2.0$  mV. For steady-state inactivation, the midpoint voltage is shifted by  $-6.0$  mV and the slope factor is not significantly changed ( $+0.7$  mV).

The obvious discrepancy in activity between BTX and C/D/E ring analogues such as **1b** has motivated additional experiments to examine ligand-receptor binding interactions. Amino acid residues that comprise the inner channel pore were selected for initial single-point protein mutagenesis experiments, following previous studies of Site II ligands such as BTX and veratridine<sup>[20]</sup> and with the aid of a homology model comprising the S5, S6 and p-loop regions of the channel (Figures 5, S3). Ten amino acids positioned on the S6 helices of domains I–IV were changed to either lysine (K) or alanine (A). All mutant channels have been previously prepared, characterized and tested against BTX.<sup>[3]</sup> In most cases, lysine modification (N434K, N784K, F1280K, F1579K, N1584K) results in an increase ( $\sim 2$ – $10$  fold) in the  $\text{IC}_{50}$  of naphthoate **1b**, consistent with destabilization of ligand binding caused by Coulombic repulsion between the charged lysine and the protonated inhibitor. Alanine mutation at three sites (F1579A, N1584A, Y1586A) also influences the ability of **1b** to block channel function, but the overall effect on  $\text{IC}_{50}$  is much less pronounced.

With the exception of the tyrosine residue at position 1586, all amino acid mutations shown in Figure 5 have been found to abrogate the effect of BTX on channel function. The marked influence of N434K, N784K, F1280K, N1579K, and N1584K on binding of both **1b** and BTX suggests some commonality in the receptor site for these two ligands. Differences between BTX and **1b** are noted, however, with two BTX-destabilizing mutations, F1236K and N1584A; against these mutant isoforms, **1b** shows a modest *increase* in affinity from that of wt- $\text{Na}_V1.4$ . Given the large volume size of the inner pore as estimated from  $\text{Na}_V$  homology models, it is possible for BTX and **1b** to bind to the open state of the channel in different spatial orientations. The lack of stereoselectivity for inhibition by either antipode of **1b** as well as the action of alkaloids such as aconitine and veratridine at Site II are consistent with an inner pore domain that can accommodate sterically large, topologically unique structures. This conclusion is further supported by docking experiments<sup>[20]</sup> of **1b** with a homology model of the channel pore<sup>[21]</sup> constructed from recent X-ray structures of bacterial  $\text{Na}_V\text{s}$ <sup>[2]</sup> (Figures 6, S4, S5). In these poses, both enantiomers of **1b** are positioned near residues N434, N784, L1280, F1579 and N1584 and the ammonium group faces the central pore, presumably blocking ion transport through charge repulsion. Future studies will examine these models in greater detail through additional mutagenesis and SAR experiments.

In sum, we have established that analogue structures of BTX possessing the C/D/E ring elements function as low micromolar inhibitors of Na<sub>v</sub> subtypes.<sup>[22]</sup> This activity is in striking contrast to the behavior of BTX as a channel agonist. Despite the evident functional differences, it appears from protein mutagenesis experiments that **1b**, and by inference related compounds, overlap the binding site of BTX in the inner pore cavity of Na<sub>v</sub>. The availability of **1b** and associated BTX structures is enabling subsequent investigations to understand how small molecules that bind the channel inner pore regulate ion gating.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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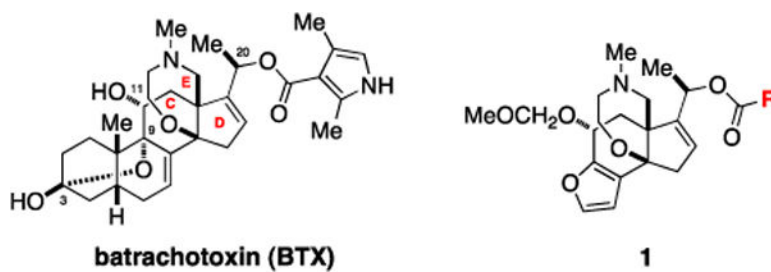
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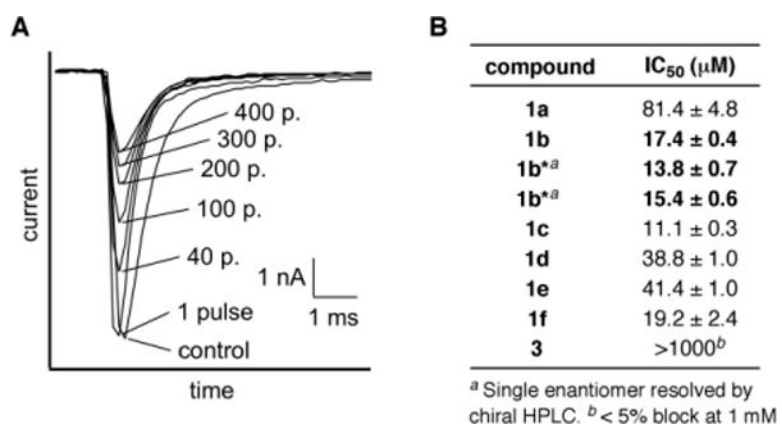
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22. Inhibition constants, K<sub>i</sub>, of the naphthoate derivative, **1b**, have been determined against l-type Ca<sup>2+</sup> (rat) and hERG channels at 4.5 and 3.2 μM, respectively. These data were obtained by the National Institute of Mental Health's Psychoactive Drug Screening Program at the University of North Carolina, Chapel Hill, see: <https://pdspdb.unc.edu/pdspWeb/for> experimental details.

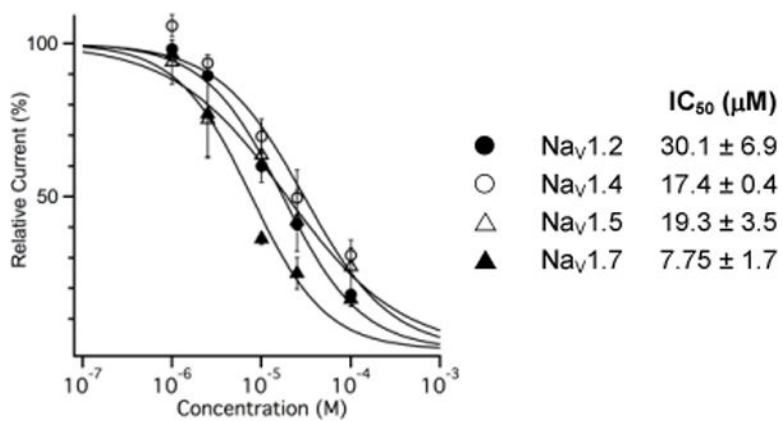




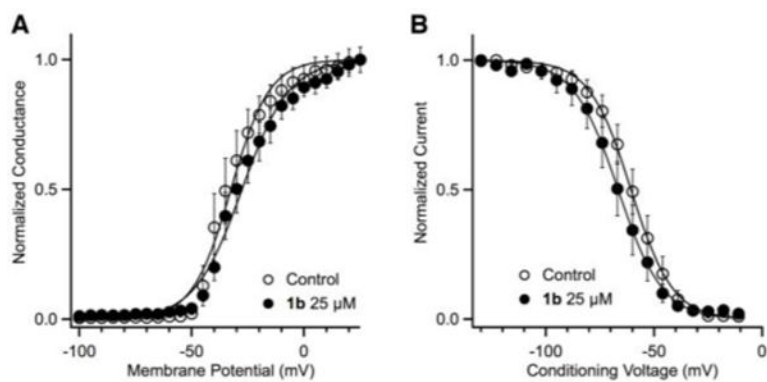
**Figure 1.**  
C/D/E ring analogues of batrachotoxin (**1**) act as potent antagonists of voltage-gated sodium ion channels (Na<sub>v</sub>s).



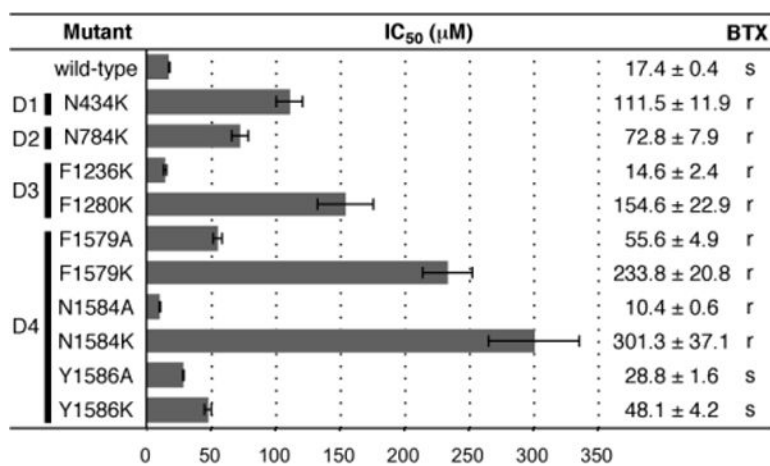
**Figure 2.** Assay results of BTX analogues against rNav1.4. (A) Use-dependent block of sodium ion current of rNav1.4 by **1b** (100 μM). Representative traces of superimposed Na<sup>+</sup> currents are labeled with the number of repetitive pulses. (B) Structure-activity relationship studies on the ester group of the C/D/E core analogues **1a–1f** and **3**.



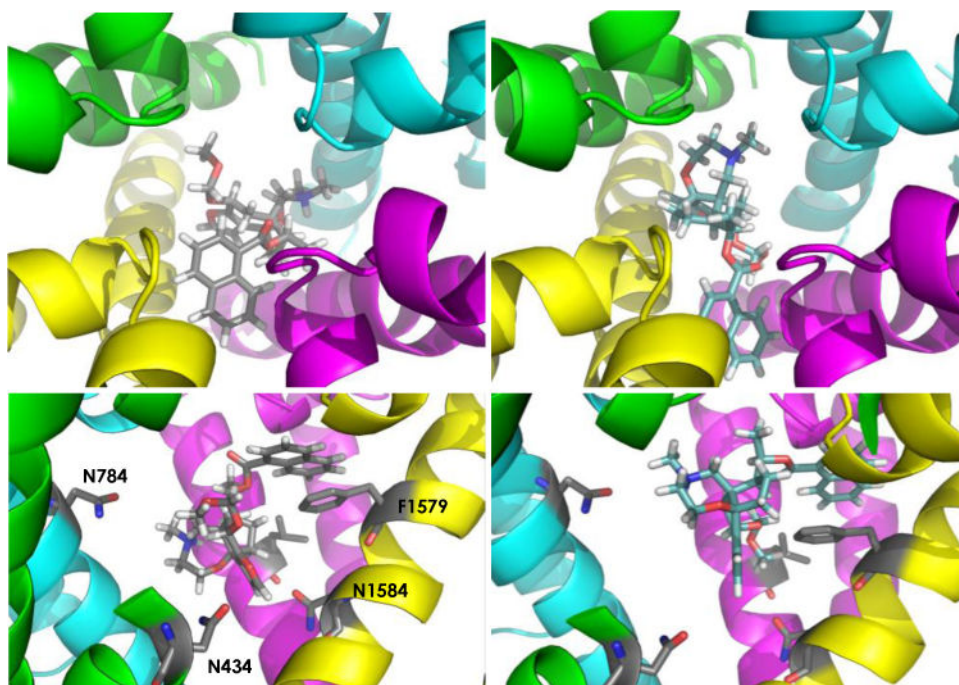
**Figure 3.** Dose-response curve of **1b** against selected  $Na_V$  isoforms. Normalized data were fit to the Hill equation.



**Figure 4.** Effects on the voltage dependence of (A) activation and (B) inactivation of rNaV1.4 by **1b** (25  $\mu$ M). The normalized data were fit to a Boltzmann equation.

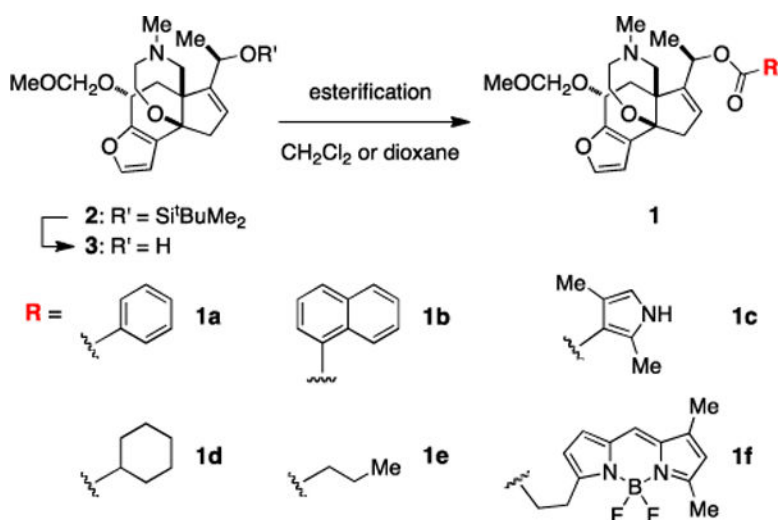


**Figure 5.** Inhibitory constants for **1b** against single-point mutants of rNav1.4 (corresponding domains are labeled D1–D4). The effect of 5 µM BTX on wild-type and mutant Nav<sub>v</sub>s is shown as s (sensitive) or r (resistant).



**Figure 6.** Enantiomers of **1b** docked to a homology model of rNa<sub>v</sub>1.4. Top panels: views down the pore axis; Bottom panels: side views (Domain I residues removed for clarity). Domain I = green; Domain II = cyan; Domain III = magenta; Domain IV = yellow. Bottom panels show residues N434, N784, L1280 (not labeled), F1579; N1584.





**Scheme 1. Synthesis of BTX analogue compounds.<sup>a</sup>**

<sup>a</sup>(a) benzoyl chloride, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 95%; (b) 1-naphthoyl chloride, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 89%; (c) (ethyl carbonic) 2,4-dimethyl-1*H*-pyrrole-3-carboxylic anhydride, *i*-Pr<sub>2</sub>NEt, 1,4-dioxane, 90 °C, 72%; (d) cyclohexanecarboxylic(ethyl carbonic) anhydride, 1,4-dioxane, 90 °C, 65%; (e) butyric(ethyl carbonic) anhydride, 1,4-dioxane, 90 °C, 75%; (f) BODIPY<sup>®</sup> FL propionic acid, 2-methyl-6-nitrobenzoic anhydride, Et<sub>3</sub>N, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 33%.