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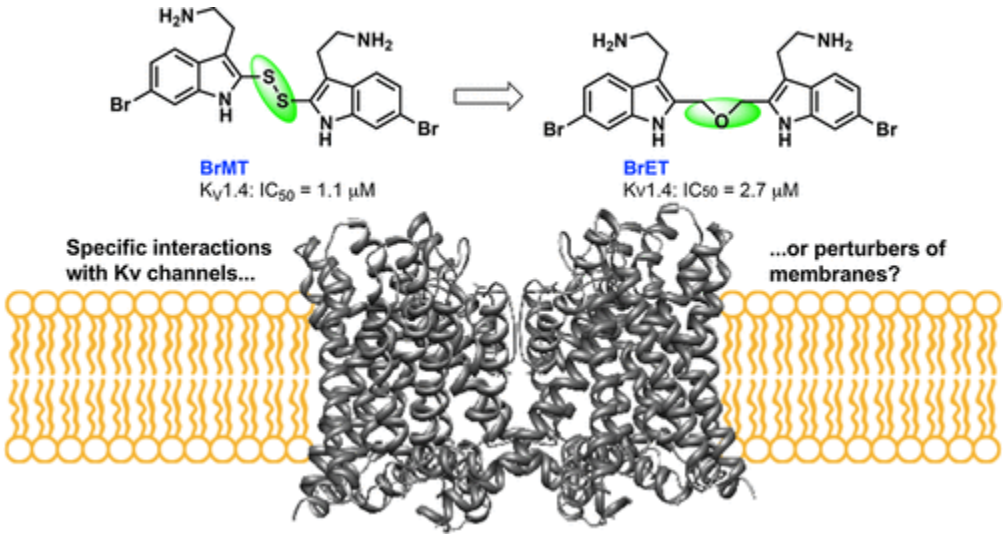
Abstract



Drugs do not act solely by canonical ligand–receptor binding interactions. Amphiphilic drugs partition into membranes, thereby perturbing bulk lipid bilayer properties and possibly altering the function of membrane proteins. Distinguishing membrane perturbation from more direct protein–ligand interactions

is an ongoing challenge in chemical biology. Herein, we present one strategy for doing so, using dimeric 6-bromo-2-mercaptotryptamine (BrMT) and synthetic analogues. BrMT is a chemically unstable marine snail toxin that has unique effects on voltage-gated K^+ channel proteins, making it an attractive medicinal chemistry lead. BrMT is amphiphilic and perturbs lipid bilayers, raising the question of whether its action against K^+ channels is merely a manifestation of membrane perturbation. To determine whether medicinal chemistry approaches to improve BrMT might be viable, we synthesized BrMT and 11 analogues and determined their activities in parallel assays measuring K^+ channel activity and lipid bilayer properties. Structure–activity relationships were determined for modulation of the Kv1.4 channel, bilayer partitioning, and bilayer perturbation. Neither membrane partitioning nor bilayer perturbation correlates with K^+ channel modulation. We conclude that BrMT's membrane interactions are not critical for its inhibition of Kv1.4 activation. Further, we found that alkyl or ether linkages can replace the chemically labile disulfide bond in the BrMT pharmacophore, and we identified additional regions of the scaffold that are amenable to chemical modification. Our work demonstrates a strategy for determining if drugs act by specific interactions or bilayer-dependent mechanisms, and chemically stable modulators of Kv1 channels are reported.

Biological membranes are composites of lipid bilayers and embedded proteins. It has long been known that membrane protein function is sensitive to the composition of the host bilayer.[(1−4)] Commonly, drugs that modulate membrane proteins are presumed to target proteins, while in fact many act by changing the bulk properties of the host bilayer, thereby altering membrane protein conformational equilibria.[(5−8)] Modulators that act by bilayer perturbation promiscuously modulate a broad spectrum of unrelated membrane proteins.[(6,7,9−15)] Upon interpretation of the mechanisms underlying the physiological actions of a drug, it thus becomes crucial to determine whether the action of an amphiphilic modulator may involve bulk bilayer perturbation, in addition to more specific interactions.

A prominent example of the importance of understanding drug mechanism involves capsaicin, a natural product of chili peppers that stimulates mammalian peripheral neurons to evoke a sensation of burning heat. Capsaicin perturbs bilayers and modulates a wide variety of membrane proteins, including Na^+, K^+, and TRP channels. Capsaicin modulates voltage-gated Na^+ and K^+ channels via lipid bilayer perturbation,[(5,7)] but capsaicin also has a specific receptor site on TRPV1.[(16)] Medicinal chemistry approaches have been successful in generating analogues of capsaicin that are selective TRPV1 inhibitors.[(17)] Similar efforts to selectively modulate Na^+ or K^+ channels with capsaicin analogues would be foolhardy, however, because modulation of membrane proteins via bilayer perturbation is fundamentally promiscuous. Thus, determining if lipid bilayer perturbation underlies modulation of a target is critical for the prediction of undesired effects on other membrane proteins.

Because lipophilic and amphiphilic drugs, by their chemical nature, partition into membranes and perturb the function of transmembrane proteins, it is a significant challenge to determine whether bilayer perturbation is the relevant mechanism underlying modulation of any particular target protein. To identify whether drugs operate by a bilayer mechanism, we previously developed a method of testing modulators for promiscuous activity against multiple unrelated classes of membrane proteins.[(7)] Although this method is effective, it requires significant resources and expertise with many membrane protein preparations. Herein, we report a greatly simplified strategy for using the structure–activity relationships (SARs) of a modulator

against a single target of interest, in combination with synthetic membrane assays, to dissect the effects of bulk bilayer perturbation from those of direct protein binding.

The medicinal chemistry target in this study is the natural product ion channel modulator dimeric 6-bromo-2-mercaptotryptamine (BrMT, **1a**). A component of the defensive mucus of the marine snail *Calliostoma canaliculatum*, it inhibits voltage-gated K^+ channels of the Kv1 and Kv4 subfamilies.[18] BrMT is an allosteric modulator that inhibits channels by slowing the voltage activation steps that precede pore opening, without blocking the central channel pore.[19,20] Allosteric modulators of Kv channels are valuable not only as research tools but also potentially as therapeutics.[21,22] BrMT itself has limited utility because it contains a chemically labile disulfide bond that is degraded by light and reducing conditions.[19] BrMT is thus an attractive target for medicinal chemistry efforts to improve its stability.

Several observations suggest that the activity of BrMT against Kv channels may be affected by nonspecific membrane partitioning. First, high concentrations of BrMT applied to outside-out membrane patches disrupt the patch clamp seal.[19] Second, a series of chimeras between the BrMT-sensitive Shaker Kv channel and the insensitive Kv2.1 channel suggest that the region imparting sensitivity is in the S1, S2, and/or S3 transmembrane regions of sensitive channels.[23] Third, the wash-in and wash-out kinetics of BrMT are multiphasic, suggesting slow accumulation of BrMT in the cell membrane during prolonged exposures.[23,24] Together, these effects are consistent with BrMT partitioning in and out of cell membranes and acting through the membrane to alter channel function. Similar to many other amphiphilic molecules that act by bilayer perturbation, the biological effect of BrMT, with its two aminoethyl groups, depends on the side of the membrane to which it is applied.[25−28] BrMT slows Kv channel voltage activation only when applied from the extracellular side of the membrane,[19] suggesting that its two positive charges may prevent it from crossing the membrane entirely. Certain Kv modulator peptides from animal venoms partition into, but do not cross, the outer leaflet of the plasma membrane bilayer. Many of these peptides bind to the transmembrane voltage sensor domains of the channels.[29−31] However, other closely related venom peptides modulate ion channels via bilayer perturbation.[32] It remains unclear whether BrMT modulates K^+ channels by direct channel binding, by perturbing the bilayer in an indirect manner, or a combination of both.[33] To elucidate the mode of action of BrMT and potentially improve its properties as a lead compound for future mechanistic or therapeutic studies, we synthesized a series of analogues, including several with stable disulfide replacements. The resulting SARs were assessed separately in membrane partitioning, perturbation, and ion channel assays to test whether specific or nonspecific interactions drive K^+ channel activities with these bis-indole compounds.

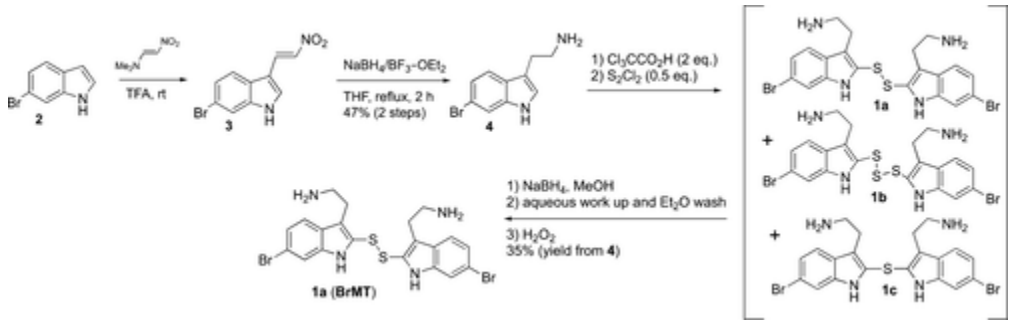
Materials and Methods

The Supporting Information contains detailed descriptions of the synthesis of all BrMT analogues, cell culture, electrophysiology, gramicidin-based fluorescence quench assay, and isothermal titration calorimetry.

Results

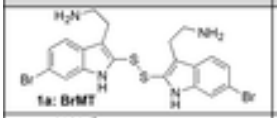
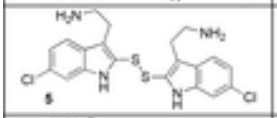
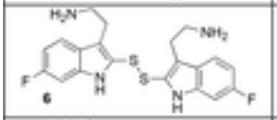
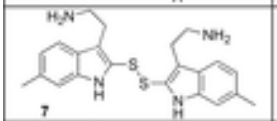
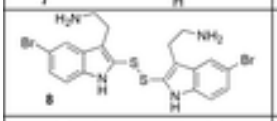
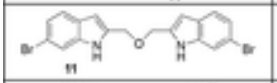
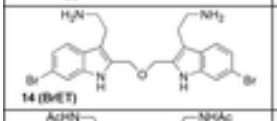
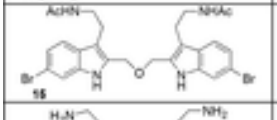
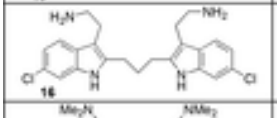
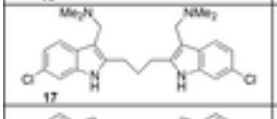
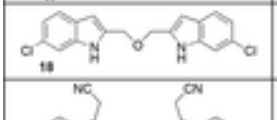
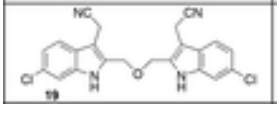
Synthesis of Novel BrMT Analogues

To determine the minimal structural features required for modulation of Kv channels with BrMT (**1a**), we designed a flexible synthesis that would enable facile modification of the tryptamine scaffold as well as the disulfide linker (Scheme 1). Our synthetic route is similar to that reported by Gallin and Hall.[34] 6-Bromotryptamine (**4**) was prepared from 6-bromoindole according to the sequence reported by Davidson.[35] Protonation of **4** with trichloroacetic acid, followed by reaction with freshly distilled S_2Cl_2,[36,37] yielded a mixture of mono-, di-, and trisulfides **1a**–**c** that was characterized by LC-MS. Using a protocol reported by Showalter for the preparation of bisindole diselenides as tyrosine kinase inhibitors,[38] we increased the yield of the desired disulfide product **1a** by treating the mixture with sodium borohydride to reduce the di- and trisulfides. Extraction of the nonpolar monosulfide **1c** with ether from the basic aqueous solution of the resulting indole-2-thiolate, followed by oxidation of the thiolate with hydrogen peroxide, gave the disulfide **1a**, which was purified by semipreparative HPLC and treated with HCl in dioxane to yield the bis-hydrochloride salt. Five different tryptamines were prepared via variations of literature protocols (see the Supporting Information for details), and these were transformed into the analogous bistryptamine-disulfides **5**–**8** (Table 1) according to the sequence of reactions in Scheme 1.



Scheme 1. Total Synthesis of BrMT

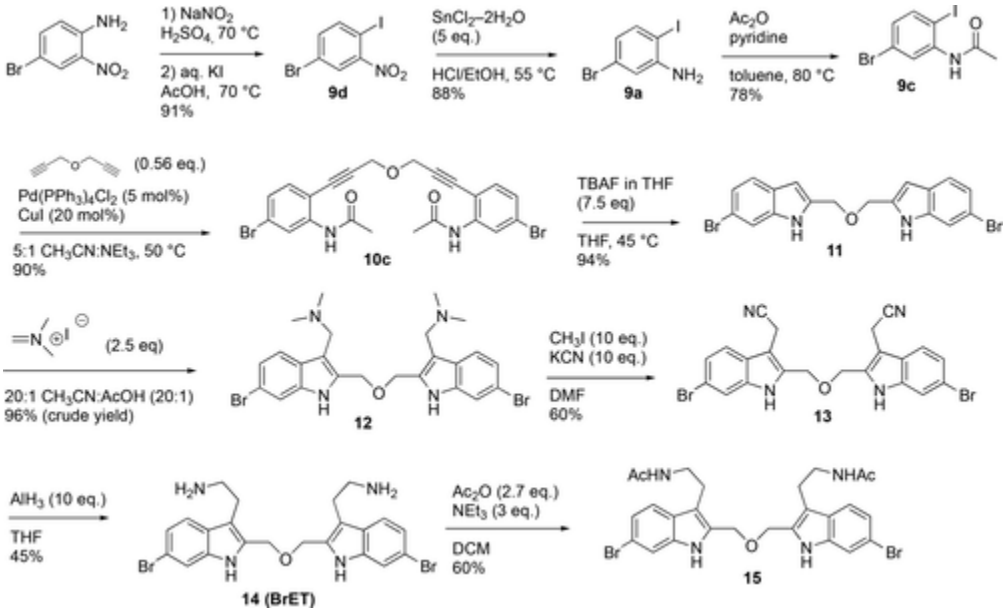
Table 1. Compilation of Properties of BrMT Derivatives

Structure	IC_{50} (μM)[a]	Hill coeff.[a]	ClogP[b]	$K_P^{W\rightarrow L}$[c]	gA_2 (μM)[d]
 1a: BrMT	1.1 ± 0.1 N = 10	1.7 ± 0.3 N = 10	5.32	560 ± 5 N = 3	40 ± 5 N = 3
 5	0.8 ± 0.2 N = 10	1.4 ± 0.3 N = 10	4.99	1000 ± 200 N = 5	13 ± 1 N = 3
 6	26 ± 9 N = 9	1.1 ± 0.5 N = 9	4.06	800 ± 200 N = 5	28 ± 1 N = 3
 7	2.1 ± 0.2 N = 21	1.8 ± 0.2 N = 21	4.81	450 ± 80 N = 4	12 ± 1 N = 3
 8	2.1 ± 0.2 N = 7	1.3 ± 0.2 N = 7	5.32	1900 ± 600 N = 4	inactive[e] (50 μM) N = 3
 11	inactive[e] (100 μM) N = 8	–	5.15	600 ± 200 N = 4	inactive[e] (50 μM) N = 3
 14 (BrET)	2.7 ± 0.3 N = 23	1.9 ± 0.3 N = 23	3.98	3100 ± 700 N = 3	2.9 ± 0.2 N = 3
 15	70 ± 30 N = 10	0.7 ± 0.2 N = 10	3.62	3900 ± 500 N = 4	1.9 ± 0.1 N = 3
 16	2.3 ± 0.3 N = 6	1.8 ± 0.2 N = 6	4.72	3300 ± 800 N = 3	12.8 ± 0.7 N = 3
 17	35 ± 7 N = 8	1.3 ± 0.3 N = 8	5.77	670 ± 90 N = 4	5.1 ± 0.3 N = 3
 18	inactive[e] (25 μM) N = 6	–	4.82	1400 ± 200 N = 4	inactive[e] (50 μM) N = 3
 19	inactive[e] (25 μM) N = 8	–	4.21	3500 ± 1100 N = 3	32 ± 5 N = 3

[a]Calculated as the best fit for inhibition of Kv1.4 ± standard deviation of the parameter fit.
[b]Calculated using ChemAxon MarvinSketch version 17.4.3.0.
[c]$K_P^{W\rightarrow L}$ is the equilibrium constant for partitioning from water to lipid. Calculated as the geometric mean ± positive standard error.
[d]Gramicidin A (gA) channel activity, as measured by the rate of quenching of intravesicular ANTS fluorescence by Tl^+. gA_2 fits are shown in Supporting Information Figure S2.
[e]Indicates IC_{50} or gA_2 was not measured (highest concentration tested in parentheses).

The relative instability of bistryptamine-disulfides, and their potential for disulfide exchange reactions in vivo,[(39)] inspired us to explore the use of alternative linkers between the indole moieties. Several two-carbon indole linkers have been reported in the literature,[(40,41)] but we expected that these would be too rigid and/or short to be effective disulfide replacements. Accordingly, we pursued a convergent synthesis of symmetrical bis-indoles by reacting suitable aniline derivatives with bis-alkynes. Dipropargyl ether was selected as our first choice, as it could provide a three-atom, ether-based linker between the two indole rings that would provide a distance between indole moieties comparable to that of the disulfide linker.

The optimized synthesis of the ether-linked compounds is given in Scheme 2. Commercially available 4-bromo-2-nitroaniline was subjected to a Sandmeyer reaction[(42)] to yield aryl iodide **9d** in 94% yield. The nitro group of **9d** was reduced to aniline **9a** using $SnCl_2$ and concentrated HCl, followed by *N*-acylation with acetic anhydride to provide the amide **9c**. Amide **9c** was subjected to the double Sonogashira cross coupling conditions (Table S1, entry 7) to yield the bis-alkyne **10c** in 90% yield. Treatment of **10c** with aqueous TBAF followed by amide hydrolysis gave **11** (see SI for details). Alkylation of the bis-indole **11** with Eschenmosher's salt[(43)] gave the bis-gramine **12** in excellent yield. Owing to the potential instability of **12**, it was converted without purification to bis-nitrile **13** upon treatment with excess iodomethane and sodium cyanide in DMF. Reduction of the nitrile groups in **13** with $LiAlH_4$ was problematic and led to partial reduction of the aryl bromide, but reduction of **13** with alane[(44)] proceeded smoothly to give the bis-amine **14** (termed BrET) in 45% yield. Acetylation of **14** with acetic anhydride furnished the bis-amide **15** in 60% yield. The related BrMT analogues **16**–**19** (Table 1) were prepared by a sequence of reactions similar to those depicted in Scheme 2. Initial attempts to reduce **19** to the corresponding diamine were unsuccessful in generating a compound of acceptable purity.



Scheme 2. Synthesis of Ether-Linker Analogues **14** (BrET) and **15**

BrMT Derivatization Alters the Potency of Ion Channel Modulation

We assessed the modulatory effects of BrMT analogues on currents through voltage-gated potassium channels. To measure the dose–response relations of many compounds in parallel, we developed an automated whole-cell voltage clamp assay against a BrMT-sensitive ion channel. BrMT inhibits Kv1 (Shaker-type) channels from invertebrates and vertebrates.[18] Among members of the Kv1 family, Kv1.4 was chosen because it is a potential target for chronic pain and lacks high-affinity modulators.[45−48] Moreover, Kv1.4 is transported efficiently and reliably to the plasma membrane in mammalian cell lines, making it optimal for automated electrophysiology.[49,50] We therefore created a CHO-K1 cell line with a tetracycline-inducible expression of Kv1.4 to provide a scalable cell culture with consistent current levels amenable to automated patch clamp methods.

Amphiphilic drugs often have variable potencies in different experimental preparations. This preparation-dependent variability may stem from membrane partitioning that is sensitive to the exact composition of the cell membrane, solution flow, and other factors.[24,51,52] Not surprisingly, BrMT has different potencies against Shaker Kv1 channels in patches and whole-cell voltage clamp of HEK cells, CHO cells, and *Xenopus* oocytes.[23] To serially compare the effects of BrMT derivatives with minimal variability, the assays were conducted with a commercially available automated patch clamp system that applied consistent solution flow for different experiments.

When BrMT was applied to voltage-clamped cells expressing Kv1.4, it inhibited the currents by slowing gating kinetics and reducing peak currents (Figure 1A). The effects of BrMT on kinetics were quantified by fitting a double exponential to the rise and decay of the current after a depolarizing voltage step (Figure 1B). Increasing concentrations of BrMT progressively slowed the gating kinetics (Figure 1C). These effects are similar to those seen with Shaker Kv1 channels.[19] BrMT has two phenomenological effects on Kv1 channels: a slowing of activation kinetics and a diminishment of peak current amplitude after a voltage step.[19] Peak current amplitudes of Kv1.4 are affected more dramatically than the rate of channel activation by BrMT (Figures 1, 2A). This greater sensitivity of the peak amplitudes may be due to a stronger coupling between activation and inactivation in Kv1.4 as compared to Shaker channels.[53] For comparison among the BrMT analogues, we therefore used the IC_{50} values determined from the changes in peak current because their fitting was better constrained than the results obtained from the more complex curve fitting procedures needed to analyze the activation kinetics. Modulation of Kv1.4 currents is apparent over a range of concentrations (Figure S1), and IC_{50} estimates were successfully obtained for most BrMT analogues (Figure 2). The Hill slopes deduced from these fits are between 1 and 2 for most analogues. Hill slopes greater than 1 could arise from cooperative interactions of the BrMT analogue with multiple subunits of Kv channels.[20] One analogue, **15**, has a lower Hill slope, which could indicate that this weakly modulating analogue acts by a different mechanism, but its low potency precludes any significant interpretations.

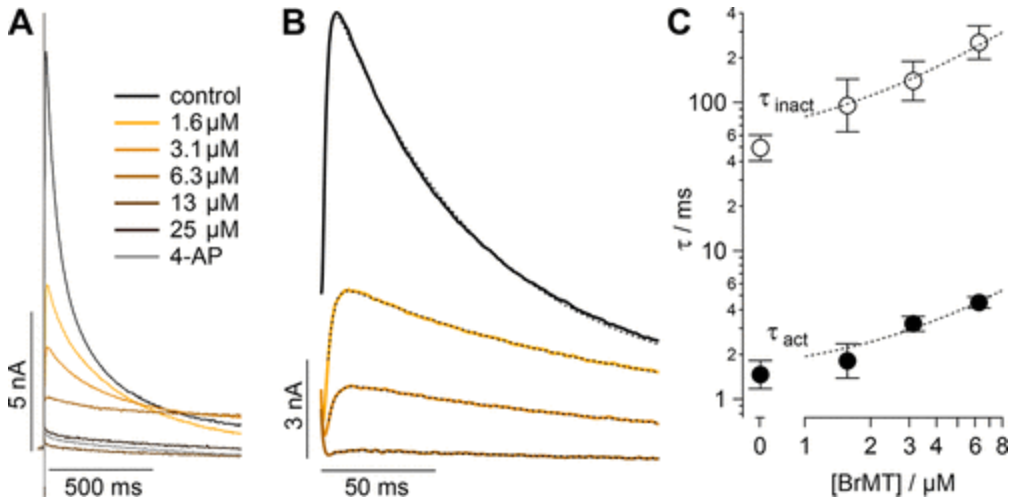


Figure 1. BrMT inhibits Kv1.4 channel activation. (A) Kv1.4 current responses to indicated BrMT concentrations during voltage steps from −100 to 0 mV. (B) Kv1.4 current responses. Colored lines correspond to the same BrMT concentrations as in panel A. Stippled lines are fits of eq 2, in the Supporting Information. Voltage steps from −100 to 0 mV. Vehicle τ_{act} = 1.72 ± 0.02, τ_{inact} = 67.3 ± 0.2; 1.5 µM τ_{act} = 2.54 ± 0.02, τ_{inact} = 136.6 ± 0.6; 3 µM τ_{act} = 3.17 ± 0.03, τ_{inact} = 181 ± 2; 6 µM τ_{act} = 4.2 ± 0.4, τ_{inact} = 300 ± 20. (C) Time constants of activation (filled circles) and inactivation (empty circles). Error bars indicate SEM (n = 9). Stippled lines are fits for Kd, as in ref [19]. K_d from τ_{act} = 2.9 ± 1.0 µM, and K_d from τ_{inact} = 1.6 ± 0.7 µM.

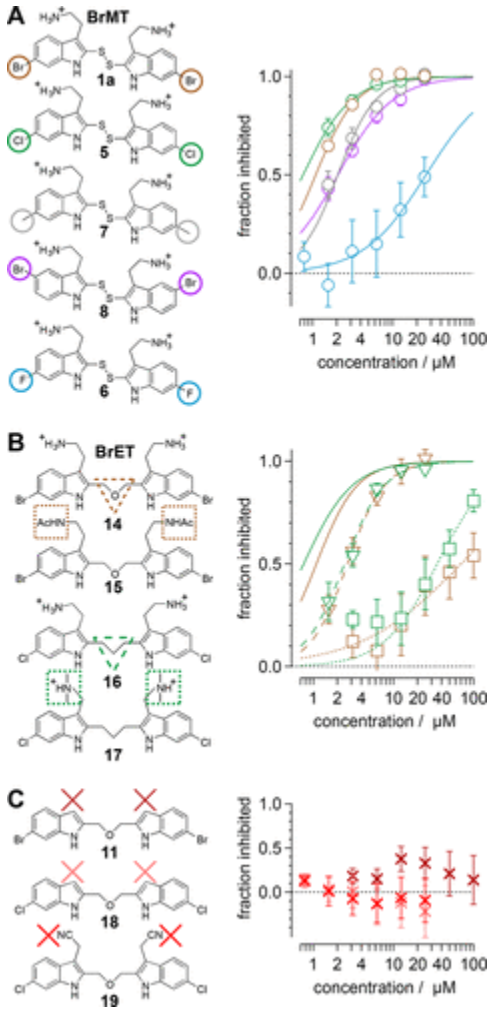


Figure 2. Structure–activity relationship of Kv1.4 inhibition. Average inhibition of peak Kv1.4 currents as a function of concentration. The shapes and colors of markers correspond to compounds with modifications indicated by shapes in the left column. Error bars indicate SEM. Lines are fits of eq 1, in the Supporting Ingormation, with parameter values indicated in Table 1. The color and pattern of lines correspond to compounds with modifications indicated by the shapes in the left column. (A) BrMT and analogues with modifications at the 6-positions on the indole rings. (B) Analogues with modifications of the disulfide linker and amines. (C) Ineffective analogues without aminoethyl groups.

Factors affecting channel inhibition emerge from analysis of SAR data. Replacement of the 6-bromo moiety of BrMT with a chloro or methyl group as in **5** and **7** had little effect on inhibitor potency, whereas substitution with the smaller and more electronegative fluoro group as in **6** led to a decrease in potency by an order of magnitude (IC_{50} = 26 μM). Prior measurements with a BrMT analogue containing only a hydride at position 6 indicated that it was also an order of magnitude less potent than BrMT against Shaker K^+ channels.(54) Moving the bromo group to the 5-position on the indole ring as in **8** had only a minor effect on potency. These results suggest that variable indole substituents are tolerated.

BrMT loses its potency against Kv channels when the disulfide is reduced to form monomeric compounds.(19) On the other hand, the disulfide linkage between the indole groups is

remarkably tolerant to replacement. For example, activity is retained when the disulfide moiety in BrMT is replaced with the ether linkage in **14** or when the disulfide in the 6-chloro-substituted analogue **5** is replaced with the trimethylene linker in **16**. The idea that alterations in the linker between the indoles have only mild effects on Kv potency was also reported by Gallin and Hall,[(34)] who found that Kv1 channel inhibition was maintained for **1a**–**c**, wherein the number of sulfur atoms bridging the indole rings was varied from one to three.

The discovery that Kv inhibition is retained with an ether or alkyl linkage between the indole rings permitted additional SAR studies using these more chemically stable scaffolds. We found that compounds **11**, **18**, and **19**, which each lack the aminoethyl side chain, are inactive at the highest concentrations tested. Moreover, the *N*-acetyl derivative **15** and the *N*,*N*-dimethylaminoethyl analogue **17** are less potent than the parent compound BrET (**14**) by more than an order of magnitude. Collectively, these results indicate that modification at the 2- and 6-positions of the indole rings of BrMT is well-tolerated, but the aminoethyl groups at the 3-positions are critical for channel activity: compounds lacking basic side chains at the 3-position were inactive (e.g., **11**, **18**, and **19**).

BrMT Derivatization Alters Bilayer Partitioning, but Partitioning Does Not Predict Ion Channel Modulation

We measured the degree to which the BrMT analogues partition into membranes using isothermal titration calorimetry (ITC).[(55)] These experiments were conducted with suspensions of large unilamellar phospholipid vesicles (LUVs) (see SI) as a surrogate for the bilayer component of cell membranes. All of the analogues partitioned into the bilayer with water → bilayer partition coefficients ($K_P^{W\rightarrow L}$) ranging between 450 and 3900 (Table 1); the calculated log([*n*-octanol]/[water]) (ClogP) values are also listed for comparison. $K_P^{W\rightarrow L}$ values increased when the disulfide linkage was replaced with a three-atom alkyl or an ether linkage. For example, replacing the disulfide linkage of BrMT (**1a**) ($K_P^{W\rightarrow L}$ = 560) with an ether linkage as in **14** ($K_P^{W\rightarrow L}$ = 3100) led to an increase in $K_P^{W\rightarrow L}$ values, as did replacing the disulfide bridge of the chloro analogue **5** ($K_P^{W\rightarrow L}$ = 1000) with a trimethylene linkage in **16** ($K_P^{W\rightarrow L}$ = 3300). If channel modulation results from compounds partitioning into the membrane bilayer, we would expect that the Kv1.4 IC_{50} values would correlate with the $K_P^{W\rightarrow L}$ values, but this is not the case (Figure 3). Examination of small structural changes that influence partitioning and/or channel modulation also indicates that the two properties are independent. Replacing the disulfide linkage of BrMT (**1a**) with the ether moiety in **14** increases $K_P^{W\rightarrow L}$ values but has little impact on IC_{50}. The acetylated, nonbasic compound **15** has an increased fraction in membranes vs its basic parent **14** ($K_P^{W\rightarrow L}$ = 3900 vs 3100), but its potency against Kv1.4 drops substantially (IC_{50} = 70 μM vs 2.7 μM) (Figure 3, brown arrows). Replacing the disulfide linkage of **5** with a trimethylene linkage in **16** also increases $K_P^{W\rightarrow L}$ values with little impact on IC_{50}. Comparison of **16** to the related *N*,*N*-dimethylaminomethyl compound **17** shows a rare example of a relatively minor structural change that simultaneously decreases both the $K_P^{W\rightarrow L}$ values and inhibitory potency (Figure 3, green arrows). Overall, increases in the partition coefficient were not coupled to an increase of inhibitory potency. Fitting a linear regression to

$K_P^{W\rightarrow L}$ and IC_{50} values did not yield any significance (r = 0.34, p = 0.37). The fraction of a BrMT analogue in the bilayer does not correlate with its potency, which suggests that bilayer partitioning is not sufficient for Kv channel modulation.

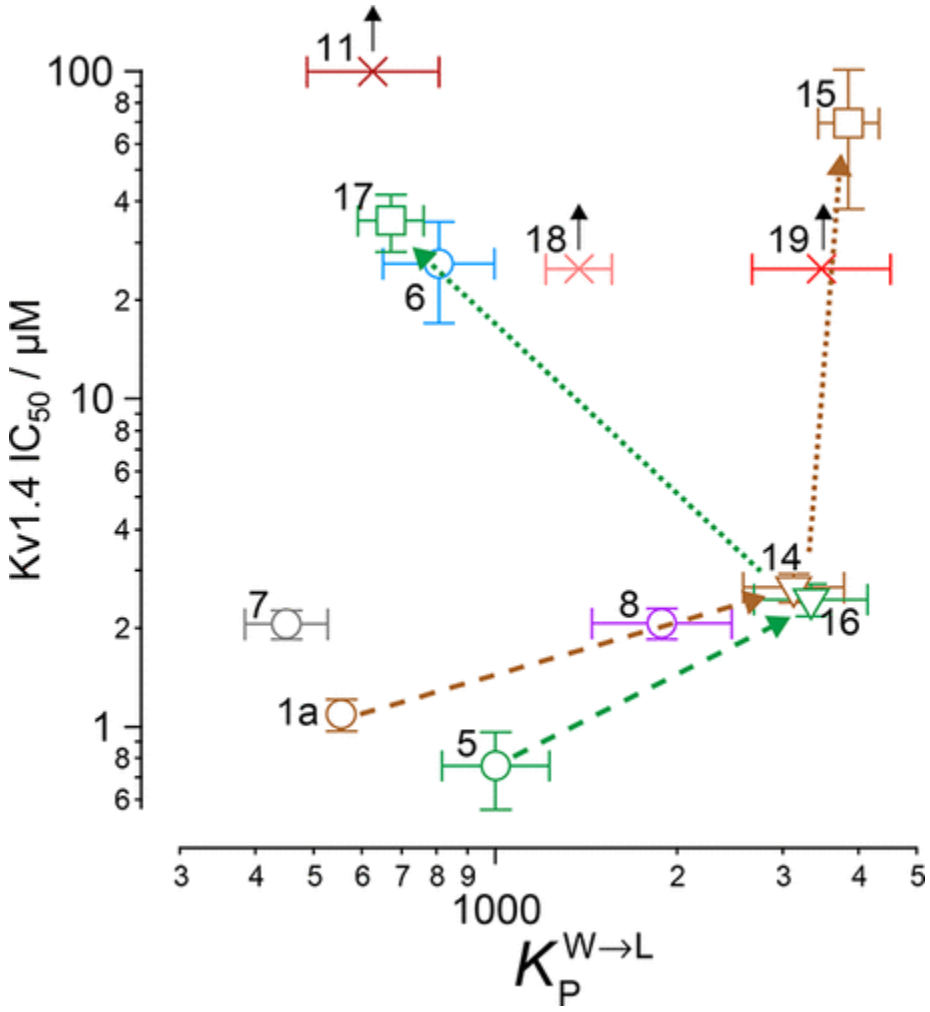


Figure 3. Membrane partition coefficients do not predict channel inhibition. Numbers by each marker denote the analogue identity. The shape and color of markers correspond to Figure 2. Values are from Table 1. Black arrows denote analogues with such a low activity that we could not measure it (null response), with the marker indicating the highest concentration tested. Brown and green arrows with dashed lines indicate modification of the disulfide linker. Brown and green arrows with dotted lines indicate modification of aminoethyl groups.

Membrane Perturbation by BrMT Derivatives Is Not Correlated with Bilayer Partitioning

Bilayer partitioning itself did not underlie the variable potencies of Kv channel inhibitors, but it remains a possibility that nonspecific bilayer perturbation might affect their inhibition of Kv1.4 because small membrane-perturbing amphiphiles are known to inhibit many Kv channels.[(7)] We therefore probed for bilayer-modifying effects using a gramicidin A (gA)-based

fluorescence assay that monitors changes of gA activity in LUVs.[56,57] This assay exploits the gramicidin channel-permeant thallous ion (Tl^+), which quenches the fluorescence of the water-soluble fluorophore 8-aminonaphthalene-1,3,6-trisulfonate (ANTS). gA permits transmembrane Tl^+ flux only as a dimer, and the equilibrium between monomeric and dimeric gA is altered by lipid bilayer perturbation. ANTS fluorescence is measured after addition of Tl^+ to a suspension of ANTS-loaded gA-containing LUVs, and the time course of fluorescence quenching (Figure 4A) provides a measure of the changes in intravesicular [Tl^+]. The initial rate of fluorescence quenching provides a measure of the initial rate of Tl^+ influx into the LUVs,[58−60] which varies with changes in the number of dimeric gA molecules in the LUV membrane. It is thus possible to determine how a drug, or other amphiphile, shifts the gA monomer/dimer equilibrium, which provides a measure of the bilayer-modifying potency of the molecule of interest. The BrMT analogue concentration that elicits a doubling of the rate of gA-dependent quenching is denoted as gA_2 and serves as a metric for the bilayer-perturbing potency (Figure S2). Note that a lower gA_2 concentration indicates more potent bilayer perturbation. Some noticeable structural trends were observed (Table 1). Analogues **11** and **18** that lack aminoethyl side chains did not have a measurable gA_2 because they caused no changes in the quench rate at the highest concentration tested. This result indicates that these analogues have minimal effects on bilayer properties. The linkage between the tryptamine monomers also affected bilayer perturbation. For example, the ether-linked derivatives **14** and **15** had the lowest gA_2, and replacing the disulfide linkage of **5** with a trimethylene chain as in **16** did not detectably change gA_2. Other changes to the aminoethyl side chains as shown by **14** vs **15** and **16** vs **17** increased the bilayer-modifying potency.

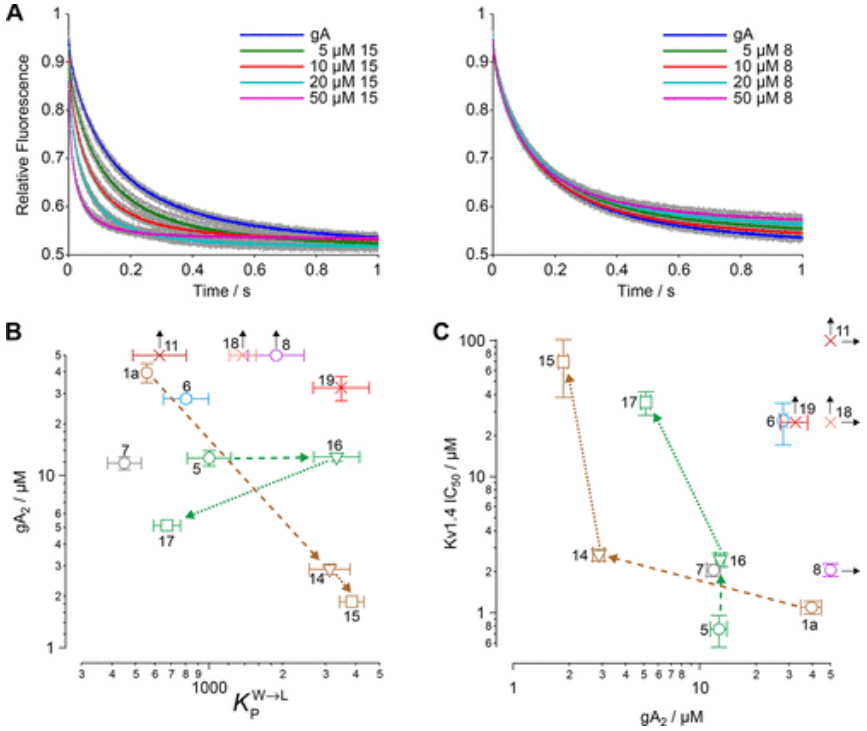


Figure 4. Membrane perturbation is distinct from partitioning and does not predict channel inhibition. (A) Gramicidin A (gA) channel activity, as measured by the rate of quenching of intravesicular ANTS

fluorescence by Tl^+. For each trace, the gray dots denote the results obtained in the nine individual repeats, whereas the colored dots denote the average value at each time point. (B) Membrane partition–perturbation relationships. Numbers next to the different markers denote the analogue's identity. The shape and color of markers are as in Figure 2. Data from Table 1. Black arrows indicate a null response, with the marker indicating the highest concentration tested. Brown and green arrows with dashed lines indicate modification of the disulfide linker. Brown and green arrows with dotted lines indicate modification of aminoethyl groups. (C) Membrane perturbation–inhibition relationship (data from Table 1).

Bilayer partitioning did not predict the bilayer-perturbing potency, as there was no obvious correlation between $K_P^{W\rightarrow L}$ and gA_2 (Figure 4B) ($r = -0.27$, $p = 0.47$). Divergent effects of structure on $K_P^{W\rightarrow L}$ and gA_2 can also be seen when comparing the related analogues **1a** vs **14** and **15**, as well as **5** vs **16** and **17**. For compounds **1a**, **14**, and **15**, a decreasing gA_2 was consistent with an increasing $K_P^{W\rightarrow L}$, as would be expected if an increase in the partition coefficient decreased the aqueous concentration needed to reach a mole fraction in the bilayer that caused the perturbation. Substituting an ether linkage into **1a** giving **14**, for example, increased bilayer partitioning and perturbation. When the aminoethyl groups in **14** are acetylated to give **15**, there is a further slight increase of bilayer partitioning and perturbation (Figure 4B, brown arrows). However, in a sequence of modifications at the same positions of the Cl-substituted disulfide in the series **5**, **16**, and **17**, gA_2 appeared insensitive to $K_P^{W\rightarrow L}$ (**5** → **16**, Figure 4B, stippled green arrows) and then anticorrelated (**16** → **17**). A particularly striking example of the divergence between gA_2 and $K_P^{W\rightarrow L}$ is seen when the bromo group at C6 in **1a** is moved to C5 in **8**, resulting in a moderate increase in $K_P^{W\rightarrow L}$ yet complete inactivity in the gA assay (Figure 4A, right panel). Overall, BrMT analogues did not show a consistent dependence of bilayer perturbation on partitioning.

Potency of Channel Modulation Is Not Dictated by Bilayer Perturbation

Bilayer perturbation was altered by BrMT analogues in a fashion distinct from partitioning, so we then assessed whether perturbation might be the mechanism underlying Kv modulation. When Kv1.4 IC_{50} values are plotted vs gA_2 values as a measure of bilayer perturbation, there is no obvious correlation ($r = -0.37$, $p = 0.36$) (Figure 4C). In fact, more potent bilayer-perturbing compounds are generally weaker inhibitors of Kv1.4. Substitution of the native disulfide linkage of **1a** for the ether linker in **14** led to a 10-fold decrease in gA_2 (10-fold increase in bilayer-perturbing potency) with a slight decrease in potency of channel inhibition (IC_{50} = 2.7 μM for **14** vs 1.1 μM for **1a**). In contrast, when the disulfide linkage in the 6-chloro analogue **5** is replaced with the trimethylene linker in **16**, there is little change in gA_2, but there is a 3-fold increase in IC_{50}. Changes to the aminoethyl side chain (e.g., **14** → **15** or **16** → **17**) decrease gA_2 but increase IC_{50} (Figure 4C). Notably, analogue **8**, which does not perturb bilayers at the highest concentration tested, is a strong modulator of Kv1.4, whereas the most bilayer-perturbing analogue **15** is a weak modulator of Kv1.4 channels. Inspection of the current traces recorded in the presence of **15** reveals that inactivation is accelerated. This action is consistent with prior findings that bilayer-perturbing detergents modulate inactivation of Kv1 channels[(61)] and suggests that Kv1.4 modulation by **15** may occur via a mechanism different from that of the less bilayer-perturbing BrMT analogues.

Discussion

Overall, these relationships show that Kv1.4 inhibition does not correlate with either bilayer partitioning or bilayer perturbation. We conclude that though BrMT and its analogues that are active against ion channels all partition into bilayers and usually perturb bilayer properties, the partitioning and alterations in bilayer properties do not drive potassium channel modulation. We conclude that specific interactions between Kv1.4 and “strong” modulators such as BrMT and BrET are critical for activity.

Limitations

Our results show that bilayer partitioning and perturbation are not critical determinants of modulation of Kv channels by BrMT or its analogues. These conclusions rely on interpretations that have caveats:

1. The bilayer partitioning and perturbation experiments were conducted with bilayers formed by synthetic phosphocholine lipids. Results in multicomponent bilayers,[(62−64)] and whole cells,[(10)] have been found to be similar to those obtained in single-component bilayers. On the basis of these previous validations, we expect the impact of compounds on single-component bilayers will extend to living cells more generally. However, our experiments do not exclude the possibility that BrMT derivatives partition differently into CHO cells and/or interact in a binding pocket with specialized lipids.

2. Our interpretation assumes that the BrMT derivatives alter Tl^+ flux through gA channels by perturbing lipid membranes, not by interacting directly with the gA channel itself. Our experiments cannot completely exclude such direct effects; results with many different structurally diverse amphiphiles show that they have similar effects on right- and left-handed channels, which effectively excludes direct interactions.[(6,8,65)] The achirality of the BrMT derivatives here precludes such a validation.

Implications

By using a series of analogues of the snail defensive toxin BrMT, we identified certain molecular features that determine its inhibition of potassium channels as well as its partitioning into and perturbation of membranes. Our findings are consistent with BrMT acting through a drug binding pocket at the lipid bilayer–potassium channel interface.[(31,66,67)] The positive charges of other amphiphilic, membrane partitioning compounds are crucial for their inhibition of Kv1 channels,[(68)] which is consistent with our observations for BrMT and analogues. Compounds **1a**, **5**, **7**, **8**, **14**, and **16** each strongly inhibit Kv channels, but of these only the native compound BrMT (**1a**) has a minimal impact on membranes. The BrMT analogue **7** is the only strong Kv1.4 inhibitor that shows a relative decrease in bilayer partitioning and is a more potent bilayer perturber. On the other hand, **8** is the only strong Kv1.4 inhibitor that is a less potent bilayer perturber and has a partition coefficient higher than that of BrMT. Thus, all of our strongly Kv1.4 inhibiting analogues either partitioned into or perturbed the bilayer more than BrMT. We speculate that membrane partitioning and perturbation may have played a role in the natural selection of BrMT to be the defensive toxin of the sea snail, possibly because of its

minimal negative impacts on snail membranes. We also conclude that bilayer interactions are not correlated with inhibition of Kv1.4.

BrMT Analogues as Research Tools

The determination of what conformations ion channels adopt is an ongoing challenge of molecular physiology. BrMT is a toxin that selectively binds to resting Kv conformations,[19] making BrMT a powerful tool to explore conformational changes of potassium channels. Conformation-selective ligands are of increasing importance for associating functional states of proteins with structurally defined conformations. For example, conformation-selective toxins were the enabling factors for reconstructing TRPV1 ion channels in an open state,[16,69−71] and co-crystals of ASIC ion channels with peptide toxin gating modifiers led to the identification of structures of new channel conformations.[72−74] In functional studies, tagged conformation-selective toxins allow imaging of Kv2 channels adopting specific conformations in live cells.[75,76] BrMT-like compounds similarly can stabilize the resting conformation of Kv1 channel voltage sensors, and they have the potential to be useful tools for studying channel conformations in cells and live tissue. The ether-linked analogue BrET (**14**) identified in this study is comparable in potency to BrMT but lacks the unstable disulfide moiety of the natural product. With the information that modifications to the indole ring and the linker between them are well-tolerated, a path is revealed for generating additional conformation-selective ligands targeting resting conformations of Kv1 ion channel voltage sensors.

Author Contributions

Chemical synthesis: C.D., M.W.D., and D.M.G. Electrophysiology: K.S.E., I.H.K., and J.T.S. Bilayer assays: O.S.A., H.I.I., R.K., T.P., and R.R. Conception of project: R.W.A., O.S.A., C.D., S.F.M., and J.T.S. Writing: O.S.A., C.D., I.H.K., J.T.S., and S.F.M.

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Notes

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Abbreviations

ACN	acetonitrile
ANTS	8-aminonaphthalene-1,3,6-trisulfonate (disodium salt)
ASIC	acid-sensing ion channel
4-AP	4-aminopyridine
BrET	BrMT analogue 2-[2-({[3-(2-aminoethyl)-6-bromo-1*H*-indol-2-yl]methoxy}methyl)-6-bromo-1*H*-indol-3-yl]ethan-1-amine
BrMT	snail toxin 6-bromo-2-mercaptotryptamine dimer
ClogP	calculated log of the partition coefficient ([*n*-octanol]/[water])
gA	gramicidin A
gA_2	concentration of compound that elicits a doubling of the rate of gA-dependent quenching
$H_P^{W\rightarrow L}$	enthalpy of partitioning from water into lipid
IC_{50}	half-maximal inhibitory concentration
ITC	isothermal calorimetry
$K_P^{W\rightarrow L}$	equilibrium constant for partitioning from water into lipid
Kv	voltage-gated potassium channels
LUVs	large unilamellar vesicles
SARs	structure–activity relationship
TRPV1	transient receptor potential vanilloid 1 channel

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00292.

- Optimization of bis-indole synthesis (Table S1), representative Kv1.4 current responses to modulators (Figure S1), gramicidin A assay data (Figure S2), and representative normalization of fluorescence in gramicidin A assay (Figure S3). Detailed methods: synthesis of tryptamine building blocks, protocol for bistryptamine-disulfide formation, protocols for synthesis of BrET (**14**) and **15**, electrophysiology methods, isothermal calorimetry methods, and gramicidin assay methods (PDF)

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