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Reversal of Tetracycline Resistance in *Escherichia coli* by Noncytotoxic *bis*(Tryptophan)s

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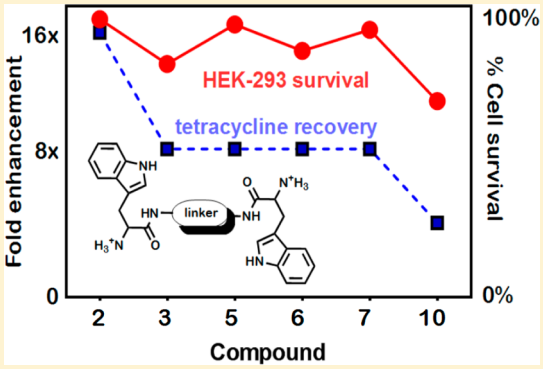
Supporting Information

ABSTRACT: Nine *bis*(tryptophan) derivatives (BTs) and two control compounds were synthesized and tested for antimicrobial activity against two *Escherichia coli* strains and a *Staphylococcus aureus* strain. The effects of linker type, shape, and conformational rigidity were manifested in dramatic differences in altering tetracycline potency when coadministered with that antibiotic. A reversal of resistance was observed for an *E. coli* strain having a TetA efflux pump. Survival of mammalian cells was assayed with good result.



INTRODUCTION

The current interest in anion binding molecules can hardly be overstated. Numerous reviews[1] of the area have appeared including two monographs.[2] Many of the anion binders derive from early work reported by Crabtree and co-workers[3] who showed that arenes having *meta*-dicarboxylic acids, e.g., isophthalic acid, form *bis*(amide)s that readily bound such spherical ions as chloride and bromide. The tris-arene hydrogen bond stabilization system was incorporated into a cryptand-like structure along with a crown ether and the combination functioned as a salt binder.[4] Multiple hydrogen bonds are available for anion stabilization in cycles such as those known as calixpyrroles.[5]

In previous work, we prepared substituted *bis*(anilide)s of isophthalic and dipicolinic acid.[6] These compounds were, like many tris-arenes, poorly soluble in water but certain of them formed channels in bilayer membranes.[7] In other, unrelated work, we found that indole could function as an amphiphilic headgroup.[8] Stable liposomes were formed from either 3- or *N*-substituted *n*-decyl- or *n*-octadecylindoles. The "head group" capability of tryptophan's indole is apparent in biology. The Leu-Trp repeats of gramicidin[9] and the tryptophans present only at the membrane interfaces in the KcsA voltage gated potassium channel[10] support this inference.

Previous work suggested that tryptophan could function effectively as an amphiphilic headgroup.[11] Our recent success with membrane active hydraphiles[12] and lariat ethers[13] as antimicrobials and as synergists for antimicrobials[14] led us to explore the biological activity of a range of tryptophan derivatives. The antibiotic health crisis[15] encouraged us to survey the activity of the *bis*(tryptophan)s (BTs), which were originally patterned as anion binders. Surprisingly, several of these novel structures inhibit the growth of Gram-negative *Escherichia coli* K-12 and Gram-positive *Staphylococcus aureus*. Even more remarkable is that at sublethal concentrations, several BTs recover tetracycline's activity against tetracycline resistant *E. coli* (Tet^R *E. coli*) expressing the tetA efflux pump. Tetracycline activity was recovered by 16-fold. Four of the molecules reported here exhibited no cytotoxicity at the minimal inhibitory concentrations (MICs) against three mammalian epithelial cell lines. A membrane disruption based activity is hypothesized based on increased permeability of Gram-negative Tet^R *E. coli* bacterial cells by one of the BTs.

RESULTS AND DISCUSSION

Tryptophan occurs in proteins with the lowest frequency of the 20 genetically coded amino acids.[16] It is hydrophobic, electron rich, and it has an N−H donor residue that can stabilize anions by hydrogen bond interactions. Tryptophan is often found in transmembrane proteins at the bilayer interface.[17,18] The frequent use of tryptophan and cationic residues in antimicrobial peptides[19] encouraged us to design BTs to assess the minimal structural elements requisite for antibacterial properties.

We screened the BTs for biological activity because we anticipated that at least some of them could be amphiphiles and show membrane activity. We evaluated the antimicrobial function of BTs using Gram positive *Staphylococcus aureus* and two Gram negative *E. coli* strains: K-12 and tetracycline resistant *E. coli*.[20] Although the antimicrobial activity observed varied according to compound structure and organism, both

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potency and selectivity of bacterial membranes over mammalian membranes was documented.

Compounds Studied. The compounds that are the focus of this report were prepared from diaminobenzenes or from α,ω-diaminoalkanes. They are shown in Figure 1. The amino

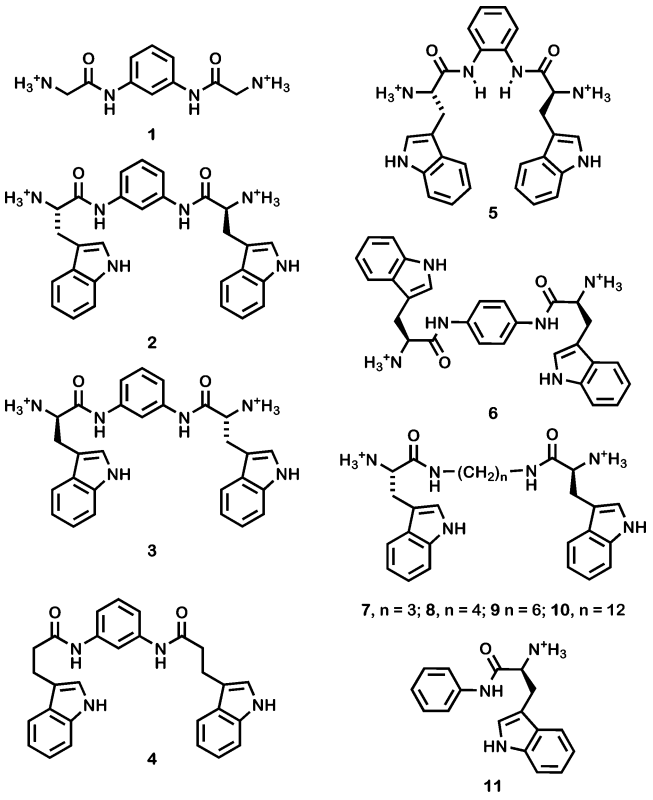

Figure 1. Chemical structures of compounds **1**−**11**.

acid, usually tryptophan, was *N*-Boc protected and the free carboxyl group was coupled with the appropriate diamine by using *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU). Four *meta*-phenylenediamine (*meta*-Ph) derivatives were prepared. They are shown in Figure 1 as **1**−**4**. Compound **1** has glycine side arms while **2** and **3** are *bis*(tryptophan) derivatives. The stereochemistry of the side arms in **2** and **3** varies: **2** = L,L and **3** = D,D. The diamine was acylated with 3-(3-indolyl)propanoic acid (IPA, sold as 3-indolepropionic acid) to form **4**. Compounds **5** and **6** are isomers of **2** but the arene is substituted *ortho* (**5**) or *para* (**6**).

Compounds **7**−**11** are related to **2** but rather than using a *meta*-phenylenediamine as the spacer or connector chain, alkyl groups link the two L-tryptophans. The alkyl groups are propylene (**7**, C_3), butylene (**8**, C_4), hexylene (**9**, C_6), and dodecylene (**10**, C_{12}). Compound **11** comprises only a part of **2** and was intended to serve as a control. Note that chloride counterions were used with all compounds except for the uncharged compound **4**.

Bacteria Used. Three strains of bacteria were the focus of this report. Two different strains of *E. coli* (Gram negative) were used. The laboratory strain of *E. coli*, K-12 (ATCC 700926), was used for preliminary MIC determinations. The tetracycline resistant strain of *E. coli* (Tet^R) was prepared by transforming competent JM109 *E. coli* (Promega) with the pBR322 plasmid (Carolina Biologicals). This plasmid contains two resistance genes. The tetA gene expresses the tetracycline resistance TetA efflux pump[21] and the amp^R gene expresses a β-lactamase enzyme[22] that cleaves the four membered ring of penicillin derivatives. The resulting *E. coli*, which we designate Tet^R *E. coli*, is both tetracycline and ampicillin resistant. The TetA efflux pump belongs to the major facilitator superfamily (MFS), spans the cytoplasmic membrane, and transports tetracycline from the cell cytoplasm to the periplasmic space.[23] This active efflux utilizes the proton gradient as an energy source.[24]

Gram-positive *S. aureus* (ATCC 29213) used for the MIC study expresses the MFS type NorA efflux pump and is methicillin sensitive. MFS type efflux pumps are clinically relevant for resistance in both Gram positive and negative bacteria.[25] We therefore used these strains to determine the MIC values for BTs and to assess their ability to recover antimicrobial potency against resistant bacterial strains.

Antimicrobial Activity. All minimum inhibitory concentration (MIC) values for compounds **1**−**11** were determined according to the methods prescribed by the National Committee for Clinical Laboratory Standards.[26] Essentially, the bacterium under study is grown to a specified optical density and added to antibiotic that is serially diluted by halves until the growth is inhibited by greater than 80%, detected spectroscopically. All the BTs were dissolved in DMSO and the solvent concentration was kept constant at 0.5% by volume in all experiments. We note that MIC concentrations are sometimes reported in μg/mL. For compound **10**, 10 μM corresponds to 6 μg/mL. We use μM here for convenience in comparisons. The MICs that are recorded in Table 1 represent

Table 1. Minimal Inhibitory Concentrations (MICs)[a]

cpd	link (AA)[b]	*E. coli* K12 (μM)	*E. coli* Tet^R (μM)	*S. aureus* (μM)
1	*meta*-Ph (Gly)	>128	>128	>128
2	*meta*-Ph (L-Trp)	64	48 ± 8	32
3	*meta*-Ph (D-Trp)	64	28 ± 4	32
4	*meta*-Ph (IPA)[c]	>128	>128	>128
5	*ortho*-Ph (L-Trp)	64	56 ± 8	32
6	*para*-Ph (L-Trp)	128	120 ± 14	128
7	$(CH_2)_3$ (L-Trp)	>128	>128	>128
8	$(CH_2)_4$ (L-Trp)	>128	>128	>128
9	$(CH_2)_6$ (L-Trp)	>128	128	>128
10	$(CH_2)_{12}$ (L-Trp)	8	10 ± 2	4
11	C_6H_5-L-Trp-NH_2	>128	>128	>128

[a]MIC resolution is in powers of 2 unless otherwise indicated by a range with ±. [b]Structure of both amino acids. [c]3-(3-Indolyl)propanoic acid.

at least two replicates of three trials each. A value of >128 μM recorded in the Table means that no growth inhibition was apparent at 128 μM so the MIC could be far higher.

Comparison between K-12 *E. coli* and *S. aureus*. The data in Table 1 show that 5 of the 11 compounds tested exhibited various levels of antimicrobial activity against *E. coli* and *S. aureus*. These compounds, **2**, **3**, **5**, **6**, and **10** are more active against Gram positive than Gram-negative bacteria. Indeed, the potency of **10** (MIC of 8 μM, 5.2 μg/mL against K-12 *E. coli*) is twice that observed against *S. aureus* (4 μM, 2.6 μg/mL). Most antibiotics are more potent against Gram-positive bacteria due to the absence of a secondary impermeable membrane.[27] Of course, a Gram-positive specific target is also possible as observed for daptomycin.[28]

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Structural Comparison. The compounds studied fall into two categories: compounds having arenyl or alkyl spacers. The compounds having aromatic spacers are **5** (*ortho*), **1**−**4** (*meta*), and **6** (*para*). The alkylene spacers range from three to 12 methylenes in **7**−**10**. Compound **11** contains a single tryptophan (no spacer) and is intended to serve as a control.

The arylene BTs are more active antimicrobials than those having alkyl spacers except for **10** [$(CH_2)_{12}$ (L-trp)], the most potent compound against the three strains of bacteria tested. Note that **11**, the single Trp control, is essentially inactive (MIC > 128 μM). Compounds **1** and **2** are identical except that the two amino acids are glycine in the former and tryptophan in the latter. Compound **2** shows modest antimicrobial activity and **1** shows none (MIC > 128 μM) against all three bacteria. The activity of **2** was also lost when tryptophan was replaced by 3-(3-indolyl)propanoic acid (**4**). Compound **4** lacks ammonium residues, but it is also achiral. Although we attribute the loss in activity primarily to the difference in charge, chirality may also play a role. Taken together, we infer that both the charged ammonium moieties and the indoles in the tryptophan residues are critical for the activity of **2**, **3**, **5**, **6**, and **10.** The disposition of the side chains in otherwise identical compounds **2**, **5**, and **6** revealed that *ortho* and *meta* substitution produced similar toxicities to the three subject bacteria, but essentially no activity was observed for *para*-phenylene *bis*(tryptophan) **6**.

A further comparison can be made between **2** and **3**, which differ only in the stereochemistry of the tryptophan residues. Both compounds showed similar activity against *E. coli* K-12 (64 μM) and *S. aureus* (32 μM). Compound **3**, in which the tryptophans have the uncommon D-configuration, was nearly twice as active (28 ± 4 μM) as the naturally occurring isomer L-tryptophan analog (**2**, 48 ± 8 μM) against *E. coli* Tet^R. Note that the MIC values in this case were narrowed from the power interval so that a closer comparison could be made. We speculate that although both **2** and **3** are similarly toxic to *E. coli* Tet^R, the D-tryptophans are metabolized less rapidly[29] and duration rather than potency is reflected in the different MICs.

The alkylene derivatives that approximate the molecular spacing of the tryptophans also show relatively low activity against all three bacteria. Thus, **7** and **8** are inactive. Compound **9** has a slightly longer spacer chain but is essentially inactive to all three bacterial strains. It is marginally more active against *E. coli* Tet^R than it is against the *E. coli* K-12 or *S. aureus*, but it is generally less active than **2** or **3** against all three bacteria. However, the greater antimicrobial activity of $(CH_2)_{12}$ (L-Trp) (**10**) compared to *meta*-Ph (L-trp) (**2**) and *meta*-Ph (D-trp) (**3**) could relate to overall separation of the ammonium or tryptophan residues. The separation of -NH_3^+ groups in **10**, the most active BT, is ∼21 Å (fully extended alkyl chain). In **2** and **3**, the separation is only ∼12 Å. Of course, the phenylene BTs are more rigid than the alkyl BTs and the conformation of **10** in particular is currently unknown.

Amphiphiles are known to enhance the permeability of bacterial boundary layers.[30] Amphiphiles are also known to form aggregates in aqueous solution. An effort to detect aggregates of **10** was made by using dynamic light scattering (DLS). Compound **10** was deemed to be the most amphiphilic (bola-amphiphilic[31]) of the structures owing to the estimated maximal spacing of the amino groups. Solutions of **10**·$(HCl)_2$ at concentrations between 10 μM and 1 mM were prepared and examined by dynamic light scattering methods. At the highest concentration, it appeared that some aggregates formed, but the counts were low and the results were considered inconclusive.

Cytotoxicity to Mammalian Cells. Our initial hypothesis was that antimicrobial activity resulted from membrane disruption. Membrane active compounds are often cytotoxic to mammalian cells.[32] The survival of three mammalian epithelial cell lines was assayed for **2**, **3**, **5**, and **10**. Inactive **6**, *para*-Ph (L-Trp) and **7**, $(CH_2)_3$ (L-Trp) were included as controls. The cell lines studied were human embryonic kidney (HEK-293), human cervix epithelial (HeLa, ATCC CCL-2), and *Cercopithecus aethiops* kidney (Cos-7, ATCC CRL 1651). Cells were cultured for 24 h in 96-well plates and treated with media containing concentrations using [MIC] and [MIC] × 2 determined previously for Tet^R *E. coli*. The number of surviving cells was determined using an XTT assay (Sigma-Aldrich); the results are represented as percent survival in Figure 2. Cells alone were used as controls and established 100% survival. The data represent two replicates of three trials and the error bars represent the standard deviation.

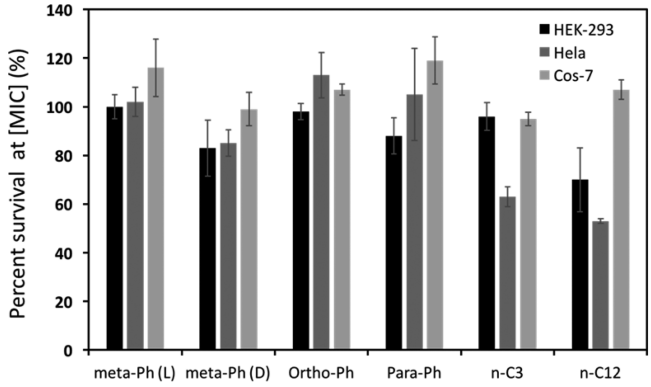

Figure 2. Cytotoxicity at the MIC concentration (against Tet^R *E. coli*) of *meta*-Ph (L-trp) (**2**, 28 μM), *meta*-Ph (D-trp) (**3**, 48 μM), *ortho*-Ph (L-trp) (**5**, 56 μM), *para*-Ph (L-trp) (**6**, 120 μM), $(CH_2)_3$ (L-trp) (**7**, 128 μM), and $(CH_2)_{12}$ (L-trp) (**10**, 10 μM) to HEK-293, HeLa and Cos-7 cells. Error bars represents the standard deviation in our results.

At MIC concentrations, arene-linked BTs **2**, **3**, **5**, and **6** showed ∼100% survival against HEK-293, HeLa, or Cos-7 cells. Alkyl-linked **7** and **10** were minimally toxic to HEK-293 or Cos-7 cells, but were moderately toxic to HeLa cells. In general, the survival of Cos-7 cells was unaffected by the highest concentration of any of the compounds tested.

The survival of all three cell lines was unaffected by a 2-fold increase in concentration of *meta*-Ph (L-trp) (**2**, 56 μM) and *ortho*-Ph (L-trp) (**5**, 112 μM), (Figure 3). In contrast, *meta*-Ph (D-trp) (**3**) at (96 μM) showed 62% survival for HEK-293 and 29% for HeLa cells. The *para*-Ph (L-trp) (**6**) at 240 μM, showed 61% survival for HEK-293 and 29% for HeLa cells. We note that the cytotoxicities for D-tryptophan (**3**) and *para*-Ph L-tryptophan (**6**) were observed at high concentrations: 96 μM and 240 μM respectively.

Two observations can be made from the data in Figure 3 concerning alkyl BTs **7** and **10**. First, **7** and **10** were more cytotoxic than phenylene BTs **2**, **3**, **5**, or **6**. At the MIC concentrations of $(CH_2)_3$ (L-trp) (**7**, 128 μM) and $(CH_2)_{12}$ (L-trp) (**10**, 10 μM), 80−100% survival was observed against HEK-293 and Cos-7 cells (Figure 2). At twice the MIC concentrations of **7** (256 μM) and **10** (20 μM), survival for HEK-293 and Cos-7 further decreased to 50−80% (Figure 3).

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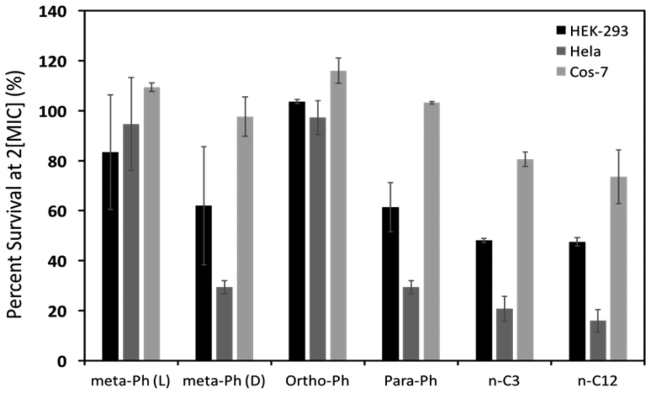

Figure 3. Graphs represents the cytotoxicity at twice the MIC concentration (against Tet^R *E. coli*) of *meta*-Ph (L-trp) (**2**, 56 μM), *meta*-Ph (D-trp) (**3**, 96 μM), *ortho*-Ph (L-trp) (**5**, 112 μM), *para*-Ph (L-trp) (**6**, 240 μM), $(CH_2)_3$ (L-trp) (**7**, 256 μM) and $(CH_2)_{12}$ (L-trp) (**10**, 20 μM) to HEK-293, HeLa and Cos-7 cells. Error bars represent the standard deviation in our results.

We infer that alkyl-linked BTs may find use as antimicrobials, although this possibility was not pursued further as part of the present effort.

Second, the cytotoxicity of $(CH_2)_3$ (L-trp) (**7**) and $(CH_2)_{12}$ (L-trp) (**10**) was greater against HeLa cells than either HEK-293 and Cos-7 cell lines. The HeLa cells are adenocarcinoma involved cervical epithelial cells. The selectivity of $(CH_2)_{12}$ (L-trp) (**10**) at 20 μM for HeLa cells over HEK-293 and Cos-7, suggests a potential application in cancer chemotherapy. While this is not the focus of the present report, we note that a $(CH_2)_8$ (L-Trp) analog of **10** prepared by Lown and co-workers showed promising cytotoxicity against 60 human cancer cell lines.[33]

Recovery of Antimicrobial Activity against a Resistant Strain. The cytotoxicity of the compounds **2**, **3**, **5**, **6**, **7** and **10** was minimal at MIC concentrations. Next, we determined whether these compounds could be used at concentrations of 1/2 MIC or lower to recover the activity of antibiotics against efflux pump expressing resistant bacteria. At these lower concentrations there should be no cytotoxicity. In addition, at the half-MIC concentrations these compounds should not have any effect on bacterial growth. We hypothesized that if certain BTs increased membrane permeability, they could recover antimicrobial potency against efflux-based resistance. This hypothesis was tested with the Tet^R strain of *E. coli* prepared in our laboratory (see above).

We determined MICs for **2**, **3**, **5**, **6**, **7**, and **10** against Tet^R *E. coli*. Compounds **6** (*para*-Ph) and **7** (n-C_3) were also included as controls. The MICs against Tet^R *E. coli* were refined compared to the power series and are reported as a range in Table 1 (above). The MICs of tetracycline and ampicillin against Tet^R *E. coli* were 900 $\pm$ 100 μM and >1000 μM, respectively. For comparison, the MIC for tetracycline against nonresistant *E. coli* K-12 is ~3 μM. Ampicillin was used to maintain selective pressure for the expression of pBR322 plasmid. Ampicillin was omitted from experiments that contained tetracycline. Next, we determined the MIC of tetracycline when coadministered with **2**, **3**, **5**, **6**, **7**, or **10**. The results are recorded in Table 2. The results are represented as the MIC of tetracycline in the presence of the indicated BTs. The fold-recovery was determined by dividing the MIC of tetracycline when used alone by the MIC of tetracycline determined in the presence of our compounds.

Table 2. Recovery of Tetracycline Activity against Tet^R *E. coli*

compounds used	[compound] μM	MIC [Tet] μM[a]	fold recovery	FIC[c]
none	0	900	n.a.[b]	n.a.
meta-Ph (L-Trp) (**2**)	24 [1/2 MIC]	56.25	16-fold	0.56
meta-Ph (L-Trp) (**2**)	12 [1/4 MIC]	112.5	8-fold	0.38
meta-Ph (L-Trp) (**2**)	14	112.5	8-fold	0.42
meta-Ph (D-Trp) (**3**)	14 [1/2 MIC]	112.5	8-fold	0.63
meta-Ph (D-Trp) (**3**)	7 [1/4 MIC]	225	4-fold	0.50
ortho-Ph (L-Trp) (**5**)	28 [1/2 MIC]	112.5	8-fold	0.63
ortho-Ph (L-Trp) (**5**)	14 [1/4 MIC]	225	4-fold	0.50
para-Ph (L-Trp) (**6**)	60 [1/2 MIC]	112.5	8-fold	0.63
para-Ph (L-Trp) (**6**)	30 [1/4 MIC]	225	4-fold	0.50
para-Ph (L-Trp) (**6**)	14	450	2-fold	0.62
n-C_3 (L-Trp) (**7**)	60 [1/2 MIC]	112.5	8-fold	0.63
n-C_3 (L-Trp) (**7**)	30 [1/4 MIC]	112.5	8-fold	0.38
n-C_3 (L-Trp) (**7**)	5	450	2-fold	0.54
n-C_{12} (L-Trp) (**10**)	5 [1/2 MIC]	225	4-fold	0.75
n-C_{12} (L-Trp) (**10**)	2.5 [1/4 MIC]	450	2-fold	0.75

[a]MIC is the observed inhibitory concentration of tetracycline in the presence of the indicated compound. MIC values represent two trials of two replicates each. MIC resolution is in powers of 2. [b]"n.a." means not applicable. [c]FIC is the fractional inhibitory concentration.

Tetracycline activity was recovered by compounds **2**, **3**, **5**, **6**, **7** and **10** at 1/2 and 1/4 of its MIC values. This recovery of tetracycline potency was based on the concentration and the structure of the compounds used. The highest recovery of tetracycline activity was observed with *meta*-Ph (L-trp) (**2**). The MIC of tetracycline was decreased from 900 μM to 56.25 μM in the presence of 24 μM of compound **2**. At twice the concentration of compound **2** (48 μM), no cytotoxicity to HEK-293, HeLa, and Cos-7 cells was apparent (Figure 2). The $(CH_2)_{12}$ (L-trp) (**10**), most potent antimicrobial in the **1**–**10** group, showed only 2 to 4-fold recovery of tetracycline activity.

The fractional inhibitory concentration (FIC) is often used as a measure of synergism or antagonism in comparing two or more compounds.[34] The FIC is the sum of the fraction of the MIC for each compound used. Synergy is defined broadly as FIC < 1, or more conservatively as FIC $\leq$ 0.5. Under the broad definition, all compounds tested can be said to have at least moderate synergy with tetracycline. All arene-based compounds fit the more conservative definition of synergy with FIC values of 0.5 or less at the tested concentrations. Compound **2** showed particularly high synergy with a FIC of 0.38. The shorter alkyl-linked compound **7** also had a FIC of 0.38, whereas the longer n-C_{12} (L-trp) (**10**) did not show synergy below a FIC of 0.75.

Since the MICs of all the compounds tested were different, we chose a single concentration to compare the efficacies of different compounds in the expectation that if any trend was apparent, it would be revealed. We compared the ability of compounds **2**, **3**, **5** and **6** to recover tetracycline activity at 14 μM, which is much lower than the MIC observed with any arene-spacer based compounds. The alkyl-spacer based compounds (**3** and **12**) were compared at 5 μM, which is much lower than the MIC observed with either compound. It is apparent from the graph of Figure 4, that at 14 μM *meta*-Ph (L-Trp), **2**, is most effective at recovering tetracycline activity against Tet^R *E. coli*. The least effective synergists were those having *para*-Ph, **6**, or propylene (n-C_3), **7**, spacers. Clearly,

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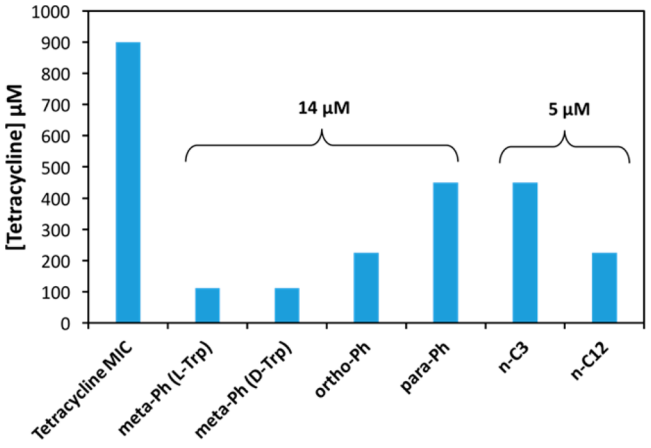

Figure 4. This graph compares the ability of *meta*-ph (L-trp) (**2**, 14 μM), *meta*-ph (D-trp) (**3**, 14 μM), *ortho*-ph (L-trp) (**5**, 14 μM), *para*-ph (L-trp) (**6**, 14 μM), $(CH_2)_3$ (L-trp) (**7**, 5 μM) and $(CH_2)_{12}$ (L-trp) (**10**, 5 μM) to recover tetracycline activity against Tet^R *E. coli*. MICs were reproduced three times and the resolution is in powers of 2.

regiochemistry and conformational mobility are contributors to the observed differences, but the precise nature of the influence(s) is not known.

Membrane Permeability. On the basis of the MIC and toxicity studies, *meta*-Ph (L-trp), **2**, and $(CH_2)_{12}$ (L-trp), **10**, have emerged as compounds of interest for different reasons. The *meta*-Ph (L-trp), **2**, shows synergy against tetracycline resistant *E. coli*, without any cytotoxicity to three mammalian cell lines. Dodecylene BT, **10**, showed the greatest antimicrobial activity, but also exhibited cytotoxicity to HEK-293 and HeLa cells. In order for the BTs to exhibit toxicity to any of the microbes, it is essential for them to penetrate the bacterial membrane. In Gram-negative organisms, the boundary membrane consists of two layers although porins are present within them that could pass these relatively small molecules.

Figure 5 shows the results of a confocal microscopy study using *E. coli* Tet^R as the test organism. The study was designed to assess the membrane permeability and viability of the *E. coli* in the presence of BTs **2** and **10**. The three panels in Figure 5 show the bright field (BF) microscopic images (top row), the result when fluorescein diacetate (FDA) is present (middle row), and the presence of propidium iodide (PI, bottom row), if any. Propidium iodide does not normally pass through boundary membranes into bacteria or other cells. When it does, it intercalates in DNA, which leads to enhanced fluorescence. Fluorescein diacetate is incorporated into the cells during growth, but is not fluorescent. If the organism is or remains vital, the diester will be hydrolyzed and fluorescein will be observed by its fluorescence emission.

The membrane permeability and viability of *E. coli* Tet^R was observed for the microbe alone or in the presence of BTs **2** or **10**. Controls for the permeability/viability assay were included for *E. coli* in the presence of a final concentration of 0.5% (v/v) DMSO (the vehicle for administration of BTs), and a final concentration of 0.1% (w/w) Triton X-100. We have recently demonstrated[35] that while small amounts of DMSO (e.g., 0.5% in media) do not alter biological activity, at higher concentrations and with certain organisms there is an effect. Thus, we never use more than 0.5% DMSO (v/v); the control is shown in the second column. Triton X-100 is a potent detergent, which is used at 0.1% or ~1,670 μM.

The images show that *E. coli* Tet^R alone or in the presence of 0.5% DMSO are vital. This is also the case when *E. coli* Tet^R is subjected to **2** at 24 μM or **10** at 6 μM. These concentrations were selected because each is 1/2 the MIC value. The lower row of Figure 5 shows that propidium iodide does not infiltrate *E. coli* Tet^R in the absence of Triton X-100, **2**, or **10**. When Triton X-100 is the adjuvant, essentially all the cells are killed and the presence of PI may simply be part of the cellular detritus. Propidium iodide fluorescence is observed when **2** or **10** is added to the cells. This indicates that the membrane permeability has increased, yet cells remain vital at the concentrations tested (cf. FDA fluorescence).

Together, the recovery of tetracycline activity and the *E. coli* membrane permeability data imply a mechanism by which membrane-active BTs overcome the efflux activity of the TetA tetracycline pump. We do not believe BTs are direct inhibitors or substrates of the TetA pump, as the fidelity of this pump to tetracycline structures is known[21] and the MIC of BTs are consistent across resistant (Tet^R) and nonresistant (K12) *E. coli* strains. Additional studies are currently underway to better understand the mechanism by which BTs recover the activity of antibiotics against efflux-based resistant bacteria.

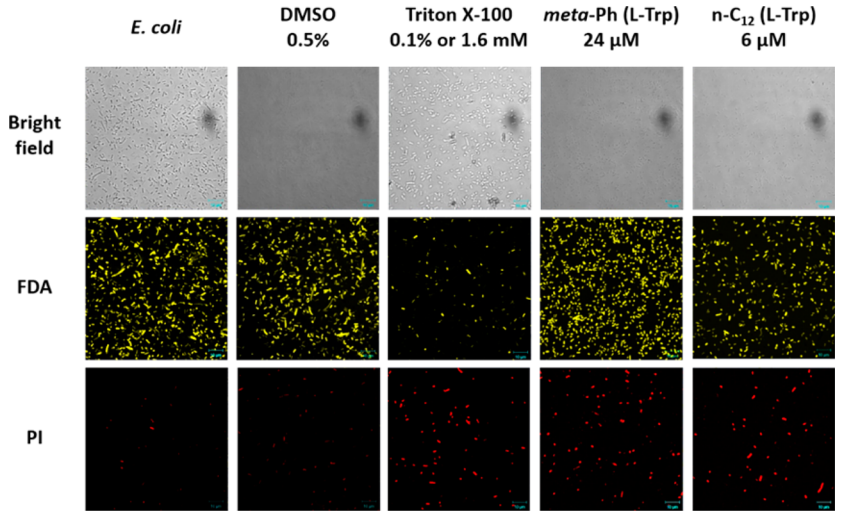

Figure 5. Tet^R *E. coli* cell membrane permeability by compounds **2** (*meta*-Ph (L-Trp)) and **10** (C_{12}-Trp) at ~1/2 MIC and controls.

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A compound that inhibits bacterial growth and penetrates into the microbe's cytosol may also penetrate into mammalian cells. We therefore conducted a similar microscopic study with the human embryonic kidney (HEK-293) cell line. In this case, only compound **2** was studied. Its activity (MIC) against all three microbes ranged from 32 to 64 μM. The microscopic study was therefore conducted at 20 μM, a value well below any inhibitory concentration, and at 80 μM, a concentration above all three MIC values. The 80 μM concentration was used to confirm the cytotoxicity of **2** and to establish the lack of serum inhibition. The results are shown in Figure 6.

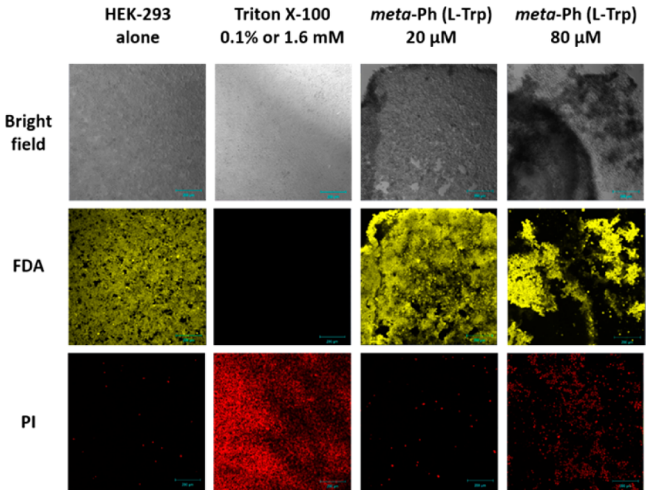

Figure 6. Mammalian cell permeability in HEK-293 in the presence of *meta*-Ph (L-Trp), **2**, at 20 μM and 80 μM.

Propidium iodide indicates an increase of the membrane permeability of HEK-293 cells and the FDA fluorescence and reports cellular vitality. When Triton X-100 is administered at 0.1% (~1670 μM), vitality is lost and a strong signal from propidium iodide reflects interaction of the dye with dispersed DNA. The results for **2** at 20 μM and 80 μM are interesting. At the lower concentration, a relatively low level of PI penetration is apparent and there is no loss of vitality. At 80 μM, there is considerable penetration of PI and some toxic effect is apparent.

These data indicate that at sublethal concentrations, *meta*-Ph (L-trp), **2**, increases the membrane permeability of *E. coli* cells, but shows no cytotoxicity or permeability alteration for HEK-293 mammalian cells. At higher concentrations, both cytotoxicity and membrane disruption are manifested.

CONCLUSIONS

A series of nine *bis*(tryptophan) derivatives (BTs) and two control compounds was synthesized and tested for antimicrobial activity. The effect of arylene and alkylene linkers on the bacteriostatic activity of the compounds was assessed against two *E. coli* strains and a pathogenic *S. aureus* strain. Structure-based studies revealed that in arylene-linked BTs the *meta* positioning of two tryptophans and the charge of the molecules are all crucial components to observe antimicrobial potency. Removal of any one property leads to loss of the antimicrobial activity. Antibacterial activity of alkylene-linked BTs was observed only for the longest dodecylene spacer. The compounds were generally more active against Gram-positive *S. aureus* than Gram-negative *E. coli*. At subinhibitory concentrations the *meta*-phenylene linked BTs recovered the antibacterial activity of tetracycline against tetracycline-resistant *E. coli*. This apparent synergy may arise from the membrane activity of these compounds as revealed by confocal microscopy. Minimal cytotoxicity was observed for the arylene-linked BTs at MIC concentrations against three mammalian epithelial cell lines. Although many amphiphilic peptides have been previously reported, this study exemplifies a minimalist structure-based approach. The simplicity of the structures elaborated in this report notwithstanding, BTs effectively reversed efflux pump-mediated resistance. With additional mechanistic and structural studies, we seek to establish a strategy for combating efflux-based antibiotic resistance with membrane-active compounds.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b05578.

Experimental procedures and spectroscopic data for all new compounds, bacterial strain information, and protocols for MICs, synergy, mammalian cytotoxicity, and membrane permeability studies are available. (PDF)

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Notes

The authors declare no competing financial interest.

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