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Evaluating the Effect of Peptoid Lipophilicity on Antimicrobial Potency, Cytotoxicity, and Combinatorial Library Design

Jeremy A. Turkett and Kevin L. Bicker*

Middle Tennessee State University, Department of Chemistry, 1301 East Main Street, Murfreesboro, Tennessee 37132, United States

Abstract

Growing prevalence of antibiotic resistant bacterial infections necessitates novel antimicrobials, which could be rapidly identified from combinatorial libraries. We report the use of the peptoid library agar diffusion (PLAD) assay to screen peptoid libraries against the ESKAPE pathogens, including the optimization of assay conditions for each pathogen. Work presented here focuses on the tailoring of combinatorial peptoid library design through a detailed study of how peptoid lipophilicity relates to antibacterial potency and mammalian cell toxicity. The information gleaned from this optimization was then applied using the aforementioned screening method to examine the relative potency of peptoid libraries against *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Enterococcus faecalis* prior to and following functionalization with long alkyl tails. The data indicate that overall peptoid hydrophobicity and not simply alkyl tail length is strongly correlated with mammalian cell toxicity. Furthermore, this work demonstrates the utility of the PLAD assay in rapidly evaluating the effect of molecular property changes in similar libraries.

Graphical abstract

*Corresponding Author: kevin.bicker@mtsu.edu.

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ORCID: Kevin L. Bicker: 0000-0002-8638-971X

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Keywords

peptoid; antimicrobial; combinatorial library; high-throughput; lipophilicity; ESKAPE

Multidrug-resistant (MDR) bacteria represent a global public health threat and significant obstacle to clinical treatment.¹ As common antibiotics lose potency against these bacteria, complications in the treatment of immunocompromised patients will result in longer hospital stays and increased mortality rates. The heightened need for novel antimicrobial compounds to combat these strains has increased interest in host-defense peptides as potential therapeutics. Antimicrobial peptides (AMPs) are integral to the immune system of many organisms.²⁻⁴ They exhibit broad spectrum activity against pathogens with minimal toxicity to mammalian cells. AMPs' mode of action is thought to be nonspecific membrane interactions, making the development of bacterial resistance unlikely. However, AMPs are prone to proteolytic instability and low bioavailability, hindering their application in a clinical setting.^{5,6}

Research into antimicrobial peptidomimetics has pursued a class of compounds which possesses the benefits of AMPs while improving upon proteolytic stability. One such alternative are peptoids, isomers of peptides in which the side chain is moved to the amide nitrogen rather than the α -carbon.⁷ This transformation is sufficient to render the compound resistant to proteolytic degradation and thus increases in vivo stability and bioavailability while maintaining antimicrobial potency. As new MDR bacterial strains are continually discovered, rapid identification of novel therapeutics is needed. To this end, we and others have explored the utility of combinatorial libraries, which allow for the rapid production of a multitude of unique compounds via split-and-pool synthesis.⁸⁻¹¹

Although synthesis of combinatorial libraries is well established, assays of these libraries to identify compounds with a desired property must be developed for each application. Our laboratory recently reported a technique that permits the rapid evaluation of large combinatorial libraries to identify peptoids with antimicrobial activity.^{8,9} The peptoid library agar diffusion (PLAD) assay relies on synthesizing duplicate strands of peptoids on a branched linker attached to a solid phase scaffold. Each strand is released by different chemical stimuli. One strand, the β -strand, is cleaved from the scaffold during screening to

assess the antimicrobial properties of the peptoid. The other strand, the α -strand, is released after screening for structure identification via mass spectrometry (MS). The PLAD assay provides swift visual readout of a peptoids antimicrobial efficacy, by zone of inhibited bacterial growth around a bead, allowing for screening of at least 400 unique potential therapeutics in a single Petri dish.

Rational library design is at the core of efficiently developing novel antimicrobial compounds. To this end, it is necessary to adapt design strategies to optimize the potency, cytotoxicity, and physicochemical properties of therapeutic compounds identified from libraries. We demonstrate here the utility of the PLAD assay in rapidly evaluating changes in library design on hit rate and antimicrobial potency. Compound lipophilicity has been shown to be central to bacterial cell membrane targeting and disruption by antimicrobial peptides and peptoids.^{12–15} Previous studies have shown lipidation of peptoids significantly increases potency against Gram-negative bacteria, while simultaneously causing the compounds to become toxic to human cells.¹² The current work seeks to further elucidate the importance of lipophilicity in the design of antimicrobial peptoid libraries. We report here the lipophilic modulation of a previously identified antimicrobial peptoid and the effect this modulation has on antimicrobial potency, mammalian cell toxicity, hemolytic activity, and physicochemical properties. Furthermore, a combinatorial library, previously observed to have no zones of inhibition in the PLAD assay, is shown to have gained modest efficacy against *Staphylococcus aureus*, *Enterococcus faecalis*, and *Acinetobacter baumannii* upon modification with a lipophilic tail. These results reinforce the importance of lipophilicity in library design and highlight the ability of the PLAD assay to quickly assess the effectiveness of changes in combinatorial library design.

The peptoid library agar diffusion (PLAD) assay reported by our lab served as the focal point for the planning stages of the current work. The PLAD assay has been used to identify compounds exhibiting antimicrobial properties against *Escherichia coli* biofilms and *Cryptococcus neoformans*.^{8,9} We chose to expand our use of the PLAD assay to the clinically relevant ESKAPE panel of bacterial pathogens (*Esch. coli*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, *E. faecalis*, and *Enterococcus faecium*). This panel of microorganisms represents bacteria that are responsible for most drug-resistant infections.¹⁶ The first step toward PLAD screening of the ESKAPE pathogens was optimization of reducing reagent conditions for each microorganism, which is responsible for release of the β -strand peptoid from the PLAD linker. An ideal reducing reagent produces a noticeable zone of inhibition around effective beads without causing deleterious effects on bacterial lawn growth compared to a control with no reducing reagent. Procedurally, the PLAD assay involves mixing together melted soft agar media maintained at 47 °C, an aliquot of the library to be screened, a reducing reagent, and an inoculant of the microorganism of interest. This mixture is then poured onto a solid agar Petri dish, allowed to cool, and incubated for an optimal amount of time to produce a lawn of microorganism on the media. The modular setup of the PLAD assay allows us to quickly and easily evaluate several reducing reagents to determine the optimal assay conditions for each microorganism. Assay screening against the ESKAPE pathogens required minimal optimization; with only *A. baumannii* and *P. aeruginosa* requiring a change in reducing agent compared to previously optimized conditions of 14mM TCEP (Figure S1). *A. baumannii* and *P.*

aeruginosa tolerance to varied concentrations of TCEP alongside two other reducing reagents, DTT and BME, was assayed (Figure S1). Optimal reducing reagent conditions for these microorganisms were determined to be 14 mM BME, which showed no deleterious effects on bacterial lawn growth.

A combinatorial tetramer peptoid library, termed JTL1 (Figure 1A), was synthesized to assess the efficacy of the PLAD assay in the varied bacteria panel. The library incorporated a mix of submonomers showing hydrophobic (isopropylamine (NVal), isobutylamine (NLeu), hexylamine (NHex)), aromatic (phenylethylamine (NPea), furfurylamine (NFur), tryptamine (NTrp)), and cationic characteristics (diaminobutane (NLys), diaminoethane (Nae)) (Figure 1B). The eight submonomers selected yielded a theoretical diversity of 4096 compounds. Split-and-pool synthesis starting with 0.5 g of TentaGel Macrobeads gave a total of roughly 33 000 individual beads, representing approximately eight replicates of the theoretical diversity. This built in replication was intentional, allowing us to in theory screen the entire diversity of JTL1 against each ESKAPE pathogen. The library was initially screened against *S. aureus*, a Gram-positive organism responsible for many nosocomial infections with excellent tolerance to PLAD assay conditions. However, screening of a significant portion of the theoretical diversity yielded no “hits,” or beads with measurable zones of inhibition (Figure 1C). This result, while not expected, was not without precedent. During concomitant experimentation in our lab using a similarly designed combinatorial library intended for screening against *Cryptococcus neoformans* H99S, one bead of 39 300 presented a zone of inhibition.⁸ Furthermore, poor efficacy was observed for this hit upon resynthesis and traditional characterization. It was postulated that the lack of potent compounds was due to low lipophilicity, consistent with studies in which long alkyl tails have been correlated with improved antimicrobial activity.¹² As such, we next sought to rapidly evaluate the utility of lipophilic moieties in JTL1 using the high-throughput nature of the PLAD assay.

As a case study, we began by investigating the effect of variable lipophilicity on the potency and toxicity of a previously identified antimicrobial peptoid, K15.⁹ Synthesis of the variant species was performed on Rink Amide resin via peptoid submonomer methods⁷ using bromoacetic acid, diisopropylcarbodiimide, and the appropriate amines. K15—13, K15—10, K15—8, K15—6, and K15—3 were synthesized using tridecylamine, decylamine, octylamine, hexylamine, and propylamine, respectively for the N-terminal submonomer (Figure 2A). K15—1, a single carbon tail variant was synthesized using traditional Fmoc solid-phase peptide methods¹⁷ with Fmoc protected sarcosine. All compounds were cleaved from the resin under acidic conditions, purified by RP-HPLC, and analyzed via mass spectrometry to confirm their respective structures (Figures S2—S7). Physico-chemical properties of the K15 variants were computationally determined by ChemAxon's MarvinSketch Calculator Plugins (Figure 2B).¹⁸ $\log D_{7.4}$, the diffusion coefficient at pH 7.4, was used as a descriptor of the variants lipophilicity at physiological pH (Figure 2B). The diffusion coefficient represents a molecule's preference for aqueous or lipophilic environments and provides predictive information about the bioavailability and partitioning of a potential drug molecule in vivo. $\log D$ is used instead of $\log P$, the partitioning coefficient, because $\log D$ factors in the ionization of a compound at a given pH, but $\log P$ does not. As would be expected, ionization of cationic or anionic groups would greatly affect peptoid solubility and diffusion. Since all of the peptoids evaluated contain ionizable

amino groups, log D values provide a more accurate representation of the lipophilicity of the compounds. These data correlate well with the percentage of less polar elution solvent (acetonitrile) during RP-HPLC purification, a second measure of hydrophobicity. Also unsurprisingly, a linear correlation was observed between log $D_{7.4}$ and alkyl tail length of the variants (Figure S8).

Antimicrobial potency of the variant compounds was determined by standard broth dilution against Gram-negative *A. baumannii* (ATCC 19606) and Gram-positive *E. faecalis* (ATCC 29212), microorganisms against which K15 had shown efficacy previously.⁹ Significant reduction in bacterial growth of both pathogens was observed only by the two peptoids with the longest alkyl tails, K15–10 and K15–13 (Table 1 and Figure S9). K15–13 exhibited minimum inhibitory concentrations (MICs) of 25 $\mu\text{g/mL}$ and 6.3 $\mu\text{g/mL}$ for *A. baumannii* and *E. faecalis*, respectively while K15–10 had MICs of 100 and 50 $\mu\text{g/mL}$ for *A. baumannii* and *E. faecalis*, respectively. K15 variants with alkyl tails shorter than 10 carbons did not exhibit any inhibition of bacterial growth even at the highest concentration tested, 800 $\mu\text{g/mL}$. The observation that Gram-positive bacteria were more susceptible to the antibacterial peptoids tested than Gram-negative bacteria is not surprising given that peptoids are hypothesized to exert antimicrobial activity through membrane targeting and disruption.²

Cytotoxicity of the K15 variants was determined against HepG2 human hepatocellular carcinoma cells and human erythrocytes (Table 1 and Figure S10). Concentrations ranging from 800 to 6.3 $\mu\text{g/mL}$ were assayed in 2-fold serial dilutions. As was observed for antimicrobial potency, alkyl tail length and lipophilicity correlated directly with toxicity. Decreased alkyl tail length resulted in decreased cytotoxicity as measured by the concentration at which 10% of the erythrocytes in the sample were lysed (HC_{10}) and concentration at which 50% of the HepG2 cells were nonviable (TD_{50}). It is important to note that a strong inverse relationship was observed between potency and toxicity. As alkyl tail length of the K15 variants increased, antimicrobial potency improved but toxicity became worse. Selectivity ratios (SR), a measurement of a compounds selectivity for pathogenic microbes over healthy cells, was calculated where possible (Table 1). The SRs calculated for K15–13 and K15–10 show narrow or no therapeutic window, seeming to effect bacteria and mammalian cells equally.

Having seen the impact of lipophilic modulation on both antimicrobial potency and cytotoxicity with a single peptoid compound, we next coupled alkyl tails of differing lengths (10 and 13) to JTL1, hypothesizing that increased library lipophilicity would increase hit rates. Recall that JTL1 was observed previously to be ineffective against bacteria in the PLAD assay. Synthesis was accomplished by coupling either tridecylamine or decylamine via submonomer methods⁷ to combinatorial peptoid libraries of identical length and submonomer composition to JTL1. This was done prior to deprotection of Boc groups from submonomer side-chain amino groups to ensure lipidation of only the peptoid N-terminus. The PLAD assay was used to screen the functionalized libraries, termed JTL1₁₃ and JTL1₁₀ (Figure 1A), along with the original JTL1 library against *S. aureus*, *E. faecalis*, and *A. baumannii* (Figure 3). The hit rate, defined as the number of beads exhibiting a measurable zone of inhibition out of the total number of beads screened, increased modestly for *S.*

aureus from 0% for JTL1 to 3.3% for JTL1₁₀ and to 4.2% for JTL1₁₃ (Figure 1B). Although little difference in hit rate between the 10 carbon alkyl tail and 13 carbon alkyl tail was observed against *S. aureus*, this was not the case for these libraries against *E. faecalis* and *A. baumannii*. For *E. faecalis*, the hit rate increased from 0% for JTL1 to 8.8% for JTL1₁₀ and to 16.7% for JTL1₁₃ while for *A. baumannii* the hit rate increased from 0% for JTL1 to 6.7% for JTL1₁₀ and to 16.6% for JTL1₁₃. For both of these microorganisms, the hit rate increased roughly 2-fold when a 13 carbon alkyl tail was used instead of a 10 carbon alkyl tail, representing the significant increase in compound potency afforded by a relatively minimal lengthening of the lipophilic moiety. Note that while zones of complete clearance of bacterial growth were observed for *E. faecalis* and *A. baumannii*, only zones of reduced growth were ever observed for *S. aureus* (Figure 3), even with multiple PLAD screenings.

The observed link between alkyl tail length, increased antimicrobial potency, and concomitant increased unwanted cytotoxicity was disconcerting to our efforts to develop antimicrobial peptoids. However, a recently submitted study from our lab reports an antifungal compound AEC5 which shares many chemical characteristics with K15–13, including a tridecyl tail, charged amino side chain, and a bulky aromatic residue.⁸ AEC5 underwent the same cytotoxic assay cell panel, but showed considerably lower toxicity compared to K15–13 and K15–10, with HepG2 TD₅₀ and erythrocytes HC₁₀ values of 56.2 and 68.7 $\mu\text{g/mL}$, respectively. With an MIC against *C. neoformans* of 6.3 $\mu\text{g/mL}$, this gave AEC5 SR values of roughly 9 against HepG2 cells and 11 against erythrocytes, representing a significantly better therapeutic window than any of the K15 variants tested here. Interestingly, the log D_{7.4} for AEC5 was calculated to be –1.81, which is much closer to that of K15–8, –1.69, than the more structurally related K15–13, 0.54. This suggests that elevated log D_{7.4} correlates more accurately than longer alkyl tail length with a higher degree of mammalian cytotoxicity, and may therefore point to overall lipophilicity as being responsible for cytotoxicity of antimicrobial peptoids, not strictly alkyl tail length.

While the potency and toxicity of the studied antimicrobial peptoids show a limited therapeutic window, the effect of alkylating a combinatorial library to obtain higher hit rates has illuminated a potential direction for further investigation into rational library design of antimicrobial peptoids. The addition of a highly lipophilic moiety to a library of compounds can effectively activate those with latent antimicrobial potential. Toxicity, while seemingly tied to degree of alkylation in the present work, might be reduced by altering the randomized peptoid submonomers in a library to include more hydrophilic residues, rather than shortening the tail. This library design would maintain the long alkyl tail that this data suggests is important for antimicrobial activity, while reducing a peptoids overall log D_{7.4}, which appears to be principally responsible for cytotoxicity.

In summary, a recently synthesized library of peptoids, JTL1, was screened against several pathogenic bacteria using the PLAD assay. No compounds exhibiting antibacterial properties were identified, even after screening several replicates of the theoretical diversity. We then sought to elucidate the necessity of a highly lipophilic moiety within an antibacterial peptoid combinatorial library and the effect of varying alkyl tail length and overall lipophilicity on activity and toxicity through modulation of the alkyl tail length of a previously identified antibacterial peptoid, K15. Lipophilicity was observed to correlate to

potency and cytotoxicity, dropping off sharply with decreased tail length. In an effort to highlight the utility of the PLAD assay in rapidly evaluating changes in library design and demonstrate the importance of lipophilicity in identifying antimicrobial peptoids, JTL1 was functionalized with 10 and 13 carbon alkyl moieties and screened against *S. aureus*, *A. baumannii*, and *E. faecalis*. Lipidation resulted in drastically improved rates of compound efficacy with visible regions of inhibited growth around the resin from which the compound was released. This information will guide development of more potent and less toxic libraries of compounds through addition of alkyl tails and adjustment of randomized peptoid building blocks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PLAD	peptoid library agar diffusion
AMP	antimicrobial peptide
MIC	minimum inhibitory concentration

Figure 1.

(A) General structures for the PLAD linked antibacterial libraries JTL1, JTL1₁₀, and JTL1₁₃. (B) Submonomers randomly incorporated into the library were chosen to display aromatic, hydrophobic, and cationic side chains. (C) Hit rate for each of the libraries against *S. aureus*, *E. faecalis*, and *A. baumannii*.

Figure 2.

(A) The structure of variants of the K15 antimicrobial peptoid identified previously with varying alkyl tail lengths on the N-terminus of the peptoid. (B) Structural, HPLC hydrophobicity, and calculated diffusion coefficient ($\log D_{7.4}$),¹⁸ and information for each of the K15 variants.

Figure 3.

Representative images of the PLAD screenings of libraries JTL1, JTL1₁₀, and JTL1₁₃ against *S. aureus*, *E. faecalis*, and *A. baumannii*. Zones of inhibition can be observed around beads displaying antimicrobial peptoids for both JTL1₁₀ and JTL1₁₃ for all three pathogens tested.

Table 1

Antibacterial Potency and Mammalian Cytotoxicity of K15 Variants^a

K15 variant	MIC <i>A. baumannii</i> (μ g/mL)	MIC <i>E. faecalis</i> (μ g/mL)	HC ₁₀ (μ g/mL)	erythrocyte/ <i>A. baumannii</i> SR	erythrocyte/ <i>E. faecalis</i> SR	HepG2 TD ₅₀ (μ g/mL)	HepG2/ <i>A. baumannii</i> SR	HepG2/ <i>E. faecalis</i> SR
K15-13	25	6.3	14.80	0.59	2.3	20.33	0.81	3.2
K15-10	100	50	188.6	1.9	3.8	147.0	1.5	2.9
K15-8	>800	>800	>800	ND	ND	365.1	ND	ND
K15-6	>800	>800	>800	ND	ND	>800	ND	ND
K15-3	>800	>800	>800	ND	ND	>800	ND	ND
K15-1	>800	>800	>800	ND	ND	>800	ND	ND

^aMIC = minimum inhibitory concentration; HC₁₀ = hemolysis concentration 10%; TD₅₀ = toxic dose 50%; SR = selectivity ratio (TD₅₀/MIC); ND = not determined.