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Determination of Pharmacokinetic Parameters of a Four-Armed, Guanidinium-Rich Structurally Nanoengineered Antimicrobial Peptide Polymer: A Research Protocol

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Abstract
Introduction: Antibacterial resistance has accentuated the need for the development of novel, efficacious pharmaceuticals. Structurally nanoengineered antimicrobial polypeptide polymers (SNAPPs) have shown promise as a candidate for a new antibacterial pharmaceutical class which may help overcome the problem of antibacterial resistance in humans or animals. Particularly, the SNAPP with a chemical formula $(RGRGRGRGRGRG)_4K_2K-NH_2$ (4R6G6) has been shown to exhibit negligible hemolysis while retaining low minimal inhibitory concentrations against bacteria. An important consideration in drug development is pharmacokinetic studies, however, there have been no prior pharmacokinetic studies involving SNAPPs. Therefore, the objective of this study is to determine the pharmacokinetic parameters of 4R6G6.
Methods: 4R6G6 will be synthesized and purified according to previously reported methods. 4R6G6 will be administered to BALB/c mice intravenously, serial blood samples will be taken from 0-48 hours after SNAPP administration and serum 4R6G6 concentrations will be quantified over time by liquid chromatography mass spectrometry. This information will be used to calculate key pharmacokinetic parameters using Pmetrics.
Results: We expect that the half-life of elimination will be on the order of hours and will exceed 3 hours, while the volume of distribution will be equal to the plasma volume of a mouse (1.2 mL/mouse)
Discussion: The half-life of elimination of 4R6G6 will influence its desirability as a future drug target. Additionally, the volume of distribution is expected to indicate a distribution into plasma, and can be used to calculate the desired dose for a certain plasma concentration in future studies.
Conclusion: Given the expected half-life of elimination on the order of hours, we expect 4R6G6 to be a strong candidate for further translational study in the race to combat antibacterial resistance. Future directions include the quantification of the safety of the drug by determining the therapeutic index *in vivo*.

Keywords: antibiotic resistance; antimicrobial peptide; dendrimer; drug development; LC-MS; pharmacokinetics; SNAPP

Introduction

Antibiotic Resistance

Antibiotic resistance (ABR) occurs when bacteria no longer respond to antibiotics designed to kill them [1]. ABR has resulted in a substantial burden to the healthcare system. According to the Council of Canadian Academies (CCA), 15 Canadians died each day in 2018 as a direct result of antibiotic-resistant infections [2]. Drug development by the pharmaceutical industry has historically focused on modifying existing antibiotic classes [3]. However, this paradigm has contributed to the ABR crisis as bacteria resistant to one antibiotic often develop resistance to other members of the same antibiotic class [4].

Background of SNAPPs

Structurally nanoengineered antimicrobial polypeptide polymers (SNAPPs) are a type of synthetic antimicrobial peptide (AMP) that takes a novel approach to combating bacteria [5]. Rather than inhibiting the synthesis of crucial bacterial biomolecules, many AMPs permeabilize bacterial membranes, leading to bacterial death via unregulated ion flux [6]. SNAPPs expand on the idea of AMPs by attaching many AMPs to a core, forming a dendrimer (a star-shaped macromolecule forming a nanostructure) which delivers a higher local concentration of AMPs to a bacterial cell [5, 7].
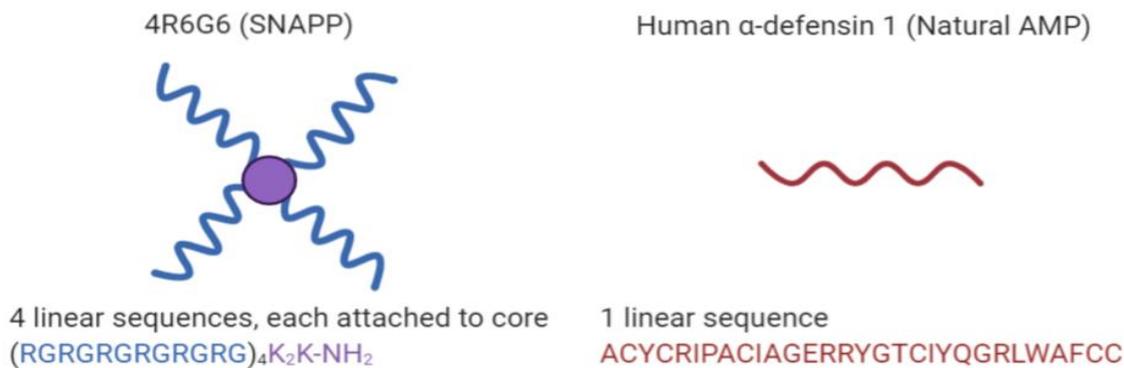
Figure 1. Conceptual comparison of 4R6G6 (a SNAPP), and human α-defensin 1 (a natural AMP). Created with BioRender. [5, 8]
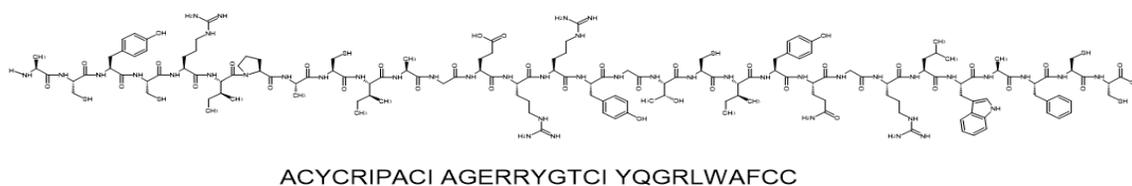
Figure 2. Chemical structure of α-defensin 1, a natural AMP. [8].
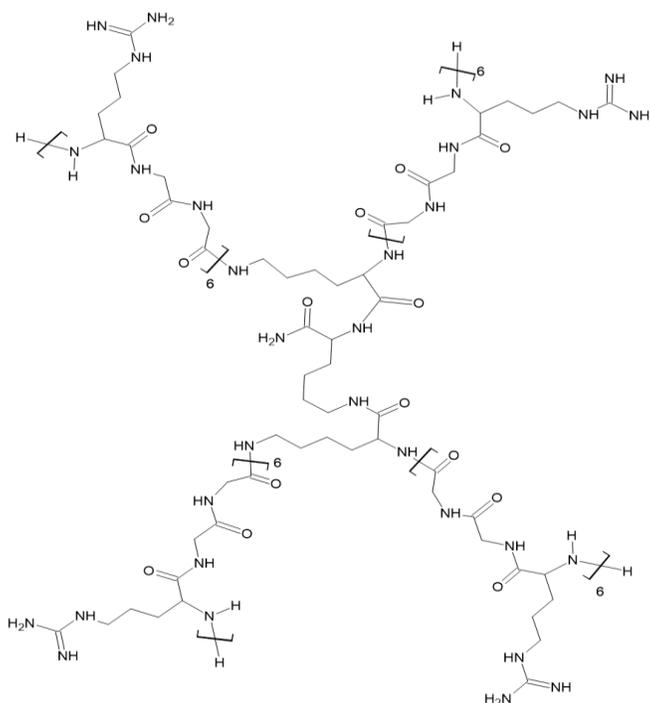
Figure 3: Chemical structure of 4R6G6, the SNAPP evaluated in this protocol [9].
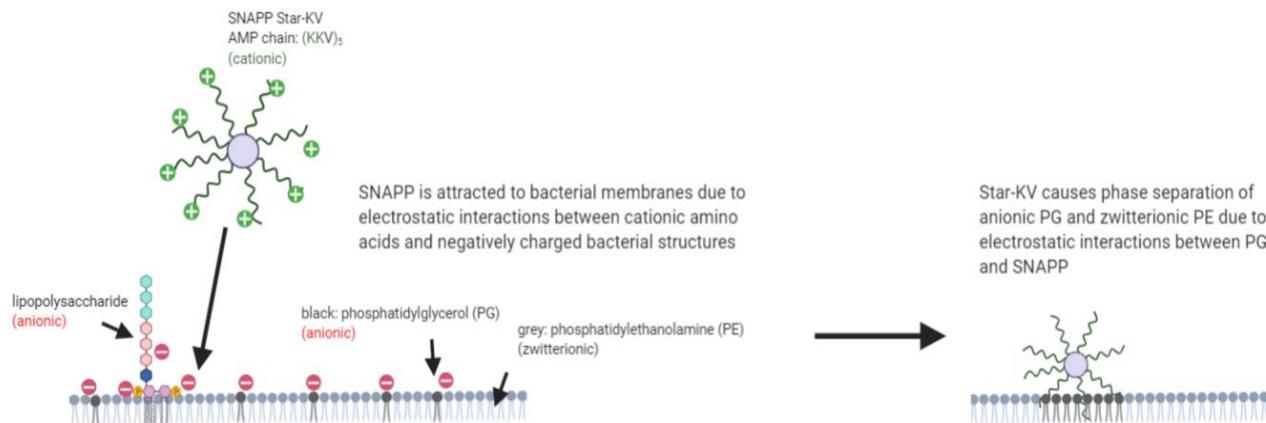
Figure 4: Electrostatic interactions between Star-KV (a SNAPP) and bacterial membranes. Created with BioRender. [10]

Mechanistic studies have shown that a Lys/Val SNAPP localizes to bacterial membranes due to electrostatic interactions between the positively charged amino acids of the SNAPP and the negatively charged structures found in bacterial membranes, including lipopolysaccharides (in Gram-negative bacteria) and anionic phospholipid heads (in all bacteria) [5]. This SNAPP-membrane interaction disrupts the bacterial membrane by inducing the separation of phospholipids in the bacterial membrane [5, 10]. An important result of this killing mechanism is that SNAPPs are likely nonspecific to bacterial strains, characterizig a broad-spectrum antibiotic [3].

Natural AMPs often cause toxicity to animals in the form of hemolysis; thus, this must be avoided in order for AMP antibiotics to have systemic applications [11, 12]. One landmark study has demonstrated that SNAPPs are effective against several strains of multi-drug resistant gram-negative bacteria (*E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii*) with no resistance observed through multiple bacterial generations [5]. This same study also validated the safety of SNAPPs by demonstrating that SNAPPs cause insignificant hemolysis in a sheep model *in vitro*.

Literature Search
A brief, non-exhaustive literature search was conducted by searching for the terms "structurally nanoengineered antimicrobial peptide polymer" and "star shaped antimicrobial peptide" on Google Scholar and PubMed. Many of the relevant articles in the literature discussed the synthesis of a variety of SNAPP and evaluated the antibacterial efficacy of that SNAPP *in vitro* [5, 9, 13, 14]. Additionally, most of these articles evaluated the toxicity of their SNAPP *in vivo*, and found that the SNAPP of interest was well-tolerated [5, 9, 13, 14]. No studies were found which evaluated the pharmacokinetics of a SNAPP.

Rationale
Briefly, pharmacokinetics is the study of the movement of exogenous compounds within the body [15]. Pharmacokinetic studies seek to determine the rates at which a drug is absorbed, distributed, metabolized, or excreted by the body. Pharmacokinetic studies therefore represent an important step in drug development, as drugs which may initially appear efficacious *in vitro* may fail *in vivo* due to poor pharmacokinetic properties. It is for this reason that we have decided to perform a pharmacokinetic study on a SNAPP.

Choice of SNAPP
Current literature has focused on optimizing the structure of SNAPPs to increase the killing efficacy against bacteria and/or decreasing toxicity towards mammalian cells, ultimately optimizing their selectivity. Therefore, we will choose a SNAPP which has demonstrated a high selectivity for the purposes of this protocol, as such a SNAPP would have the greatest clinical utility. A recent study by Wang *et al.* (2020) has reported a 4-armed, guanidinium-rich SNAPP with a low minimal inhibitory concentration (MIC) against both Gram-positive and Gram-negative bacteria, while exhibiting exceptionally low hemolysis against rat red blood cells *in vitro* [9]. The reported selectivity of this guanidinium-rich SNAPP is significantly higher than the reported selectivities of a different class of SNAPPs consisting of poly(L-lysine) chains grafted to a branched polyethylenimine core (PEI-*g*-PLL) [13]. An additional advantage of 4-armed SNAPPs such as the ones reported by Wang *et al.* (2020) is that they are easier to produce than SNAPPs with a greater number of arms [14]. Thus, we will choose to evaluate the pharmacokinetic parameters of the SNAPP with a chemical formula of $(RGRGRGRGRGRG)_4K_2K-NH_2$ (4R6G6) [9]. The chemical structure of 4R6G6 may be found in Figure 2.

Methods

SNAPP Synthesis

4R6G6 will be synthesized using methods described by Wang *et al.* (2020) [9]. Conceptually, the 'core' of the structure, containing four primary amines, will be created by coupling lysine to a resin, then coupling two more lysine molecules to the two free amine termini of the resin-bound lysine. Then, the 'arms' of the structure will be created using sequential additions of glycine and arginine. The completed dendrimer will be then cleaved from the resin and purified using high-performance liquid chromatography (HPLC) following the conditions described below. Electrospray ionization mass spectrometry (ESI-MS), and H-NMR will be used to confirm the successful synthesis of 4R6G6. Following confirmation, the 4R6G6 solution will be lyophilized to yield a solid.
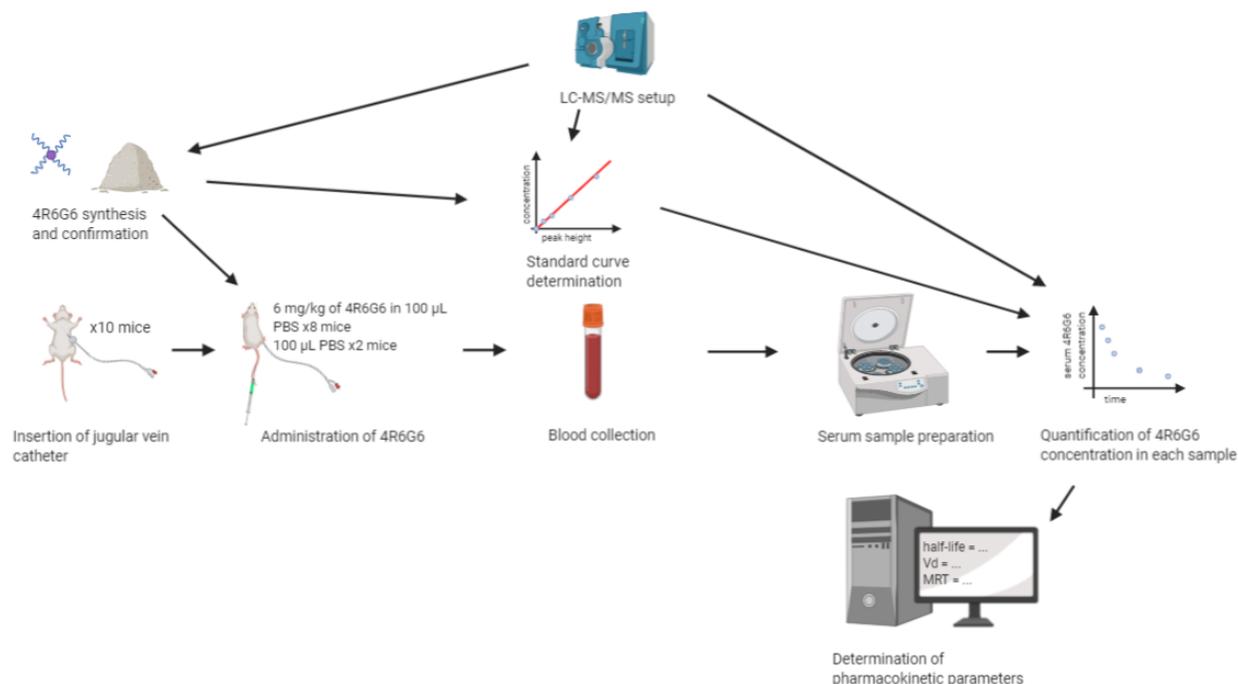
Figure 5: Schematic of experimental protocol. An arrow originating from one step and pointing to another indicates that the completion of the latter step is dependent on the former step.

Conditions for Mass Spectrometry

To determine a suitable ion transition to monitor, ESI-MS will be performed on a SNAPP solution produced by mixing 4R6G6 with 0.1% trifluoroacetic acid (TFA) in water to a final concentration of 10 mg/mL [5, 9, 16]. Suitable transitions will produce ions which appear as local maxima on product ion plots. A triple quadrupole mass spectrometer will be operated in multiple reaction monitoring mode with a positive ion electrospray source [16]. The initial MS parameters will be as follows: capillary voltage set as 4.5 kV, collision energy set as 35.0 V, collision cell exit potential set as 10.0 V, which are within the range of parameters used by Chen *et al.* (2018). These parameters may be further optimized by modifying the parameters with a goal of obtaining transitions with the largest signal-to-noise ratio on the product ion plot produced by a mass spectrometer, resulting in a more precise estimate of SNAPP concentration. According to the ESI-MS spectra published by Wang *et al.* (2020), the 4R6G6 mass spectra is expected to have strong peaks resulting in the formation of ions at 691.00, 614.35, and 789.50 M/Z [9].

Conditions for LC-MS

To quantify peptide concentration, liquid chromatography mass spectrometry (LC-MS) will be used [17]. 4R6G6 concentrations will be measured by LC-MS, consisting of a HPLC system coupled to a triple quadrupole mass spectrometer [9]. The mobile phases will consist of 0.1% TFA in water (mobile phase A) and 0.1% TFA in acetonitrile (mobile phase B). Peptides will be bound to a HPLC column at 40°C for 0.5 minutes with 100% mobile phase A, then eluted with a linear gradient of 5 to 100% mobile phase B for 40 minutes at a 1 mL/min flow rate. Peptide solutions eluted to mobile phase B will be processed with MS. To purify the synthesized SNAPPs, a 16 mL/min flow rate will be used following the above parameters.

Determination of a Standard Curve

The desired upper limit of quantification is 200 μg/mL, which is two times greater than the maximum possible concentration of 4R6G6 at the time of the first blood collection at 2.5 minutes (assuming that the 4R6G6 will be sufficiently distributed in the bloodstream by this point) [9, 18]. 6 standard solutions of concentrations 6.3, 12.5, 25, 50, 100, and 200 μg/mL will be produced by mixing 4R6G6 with 0.1% TFA in water. MS will be performed on each standard solution. A standard curve will be created through linear regression of the known 4R6G6 concentration against the peak height for the ion transition determined previously. The limit of detection (LOD) will be computed as 3 SD/b, where SD represents the standard deviation of the y-intercept of the regression line, and b represents the slope of the calibration curve [19].

Administration and Delivery

10 wild-type BALB/c mice, maintained under pathogen-free conditions will be used at 8 to 10 weeks of age. 6 mg/kg of 4R6G6 in 100 μL of phosphate-buffered saline will be administered via intravenous injection to the lateral tail veins of a group of 8 mice [9, 13, 14]. The lateral tail vein injection will be done using a 27-30 gauge needle towards the direction of the head, as established in a protocol by the American Association for Laboratory Animal Science [20]. As a vehicle control, 100 μL of phosphate-buffered saline will be administered intravenously to 2 mice in the same manner [14].

Blood Collection

Similarly to Lei *et al*. (2018), blood samples will be collected at 2.5, 5, 10, 20, 30 minutes, 1, 2, 4, 8, 12, 24, 36, and 48 hours, after initial administration of the SNAPP formulation [21]. However, as this is a repeated blood collection, blood will be collected from the jugular vein through the implantation of a catheter according to the protocol published by Park *et al.* (2018) [22]. Briefly, mice will be anesthetized through the administration of an inhalation agent. Hair near the area of catheterization will be removed, and the location of the right external jugular vein will be identified. Incisions will be made to expose the vein, a suture will be slid underneath, then tied around the vein. A catheter will be implanted by making a small incision in the wall of the vein, then sliding the tip into the vein. Blood samples will be taken by connecting a line of tubing to the implanted catheter.

Preparation of Serum Samples

Blood serum sample preparation will be performed according to a protocol previously established by Lei *et al*. (2018) [21]. Blood samples will be centrifuged at 3,000 rpm for 10 minutes to obtain serum samples. 0.5 mL of 5% acetic acid in acetonitrile will be added to 200 μL of the serum. This mixture will be vortexed for 2 minutes, then centrifuged at 12,000 rpm for 10 min. The aqueous phase will be removed and dried under nitrogen in a water bath at 50°C. 200 μL of 0.1% TFA in water (mobile phase A) will be used to dissolve the resulting sample. The final samples will be filtered through membrane filters with a 0.22 μm pore size, and analyzed with LC-MS using the protocol described above. Serum SNAPP concentrations will be quantified by inputting the peak height into the formula of the standard curve.

Pharmacokinetic Analysis

The pharmacokinetic parameters area under the curve (AUC), mean retention time (MRT), body clearance (CL), volume of distribution (V_d), and half life of elimination (T_{half}) will be obtained using the makeNCA command from the Pmetrics package in R [23]. The pharmacokinetic parameter K_e will be calculated as $\ln(2)/T_{half}$.

Results

It is expected that the SNAPP synthesis will produce the expected product with a high degree of purity, as this synthesis procedure was used by Wang *et al*. (2020) to obtain a product with 96.3% purity [9]. It is expected that the mass spectrometry with the aforementioned parameters will result in product ion transitions with the formation of ions at 691.00, 614.35, and 789.50 M/Z [9]. The correlation coefficient for the LC-MS standard curve should be close to 1 to ensure precision of the results [24]. Based on the *in vitro* degradation study of a different SNAPP (PEI-*g*-PLL) conducted by Lu *et al.* (2019), T_{half} is expected to be on the order of hours, and is expected to exceed 3 hours but is not expected to exceed 24 hours [13]. V_d is expected to be approximately equal to the plasma volume in rats (1.2 mL/mouse). The control mice are expected to have a 4R6G6 level below the LOD in the blood, as 4R6G6 should not be present in the controls.

Discussion
Design Choices

The protocol described will allow for the determination of pharmacokinetic properties of SNAPPs administered intravenously in a murine model. The SNAPP synthesis procedure used by Wang *et al.* (2020) was the preferred method of producing 4R6G6, as it had been previously shown to produce 4R6G6 of high purity [9]. LC-MS was chosen as a method of determining the serum 4R6G6 concentration as it had previously been used to accurately and precisely determine the serum concentration of a different AMP [16]. The concentrations of the standards for the standard curve were chosen such that the standard curve could be shown to be linear across several orders of magnitude of concentration. BALB/c mice, the most widely used inbred strains in animal experimentation, were selected as the model organism as they are commonly used to test drugs which are not yet known to be safe in humans [25, 26]. We chose to evaluate the pharmacokinetics of intravenously administered 4R6G6, as intravenously

administered AMPs are generally preferred because of their longer elimination half-life (ranging from hours to days) compared to AMPs administered through other routes [27]. The dose of 6 mg/kg for the experimental group was selected as it has been previously found to be a safe and effective dose for 4R6G6 by Wang *et al.* (2020) [9]. The catheterization procedure is required as it is one of the few methods able to withdraw serial blood samples within a relatively short period of time without harming the mice [28]. The time points for blood collection were selected to be similar to the time points used in a similar study by Lei *et al.* (2018) researching the pharmacokinetics of Piscidin-1, another AMP with a half-life on the order of hours [21]. There is a shorter duration between blood collections immediately after administration, as it is expected that the blood concentration of SNAPPs would change the most during this time.

Significance

V_d is a pharmacokinetic parameter representing the apparent volume in which the drug resides in the body [29]. If the measured V_d is similar to the volume of plasma in mice (1.2 mL/mouse) as expected, this would confirm that the drug is distributed in the bloodstream [13]. Clinically, the V_d may be used to determine the appropriate intravenous drug dosage based on a desired drug concentration with the formula $dose = C_p V_d$, where C_p represents the desired plasma drug concentration [30]. The measured AUC may be used in future studies as part of the measurement of bioavailability when examining another form of administration as calculated by the following equation: bioavailability = AUC_{other}/AUC_{IV} [31].

T_{half} is another important pharmacokinetic parameter to optimize. It is generally accepted that elimination of drugs follows first-order exponential decay [15]. T_{half} therefore represents the time it takes for 50% of a drug to be removed from the body. A T_{half} which is too long may result in complications due to the accumulation of drug in the system, whereas a T_{half} which is too short may necessitate frequent dosing to obtain a desired steady-state concentration [32]. It has been suggested that a dosing interval for intravenous administration can be approximately once per half life (for example, once per day for a drug with a half-life of 24 hours) [33]. It is expected that T_{half} should be greater than 3 hours, as it was previously shown that a PEI-*g*-PLL SNAPP was degraded by less than half after 3 hours of incubation with the protease trypsin (which likely represents much faster degradation than would occur in the body) [13]. It is also expected that T_{half} should be less than 24 hours, as the PEI-*g*-PLL SNAPP was completely degraded after 24 hours under the same conditions.

Limitations

There exist numerous possible variations on this research which will not be explored in this proposal.

Several different types of SNAPPs exist, which may vary in their pharmacokinetic properties. Different methods of administration and the use of different vehicles of administration may also differ in their observed pharmacokinetic properties [34]. Additionally, the mechanism of the metabolism of SNAPPs has not yet been explored, although we hypothesize that SNAPPs might be metabolized similarly to other AMPs.

Conclusions

Given the absence of literature on the pharmacokinetic properties of SNAPPs, this study outlines a protocol that can be used to determine the pharmacokinetic parameters of the SNAPP 4R6G6 administered intravenously. We propose preparing and administering 4R6G6, then determining the pharmacokinetic parameters by observing the serum SNAPP concentration over time with LC-MS. It is hypothesized that these parameters will be on the order of hours, which will provide an indication that SNAPPs may be suitable drug candidates. This study has the potential to advance the development of a new drug which may combat ABR. This design involving a murine model allows for fast, preliminary study results. However, future translational studies will be needed to confirm the pharmacokinetics of 4R6G6 in humans or other animals.

SNAPPs are a promising target for combating future antibiotic resistance. However, further research is required in order to achieve the goal of determining the viability of SNAPPs as a drug class. In this protocol, we propose an important initial step, the determination of pharmacokinetic parameters in a mouse model. However, there is still much work to be done to achieve this goal. For example, one other important step is the determination of the safety of the drug *in vivo*, by measuring its therapeutic index. Several other studies have evaluated the safety of SNAPPs, for example, by performing hemolysis assays as quantitative toxicology measures [9, 13]. However, none have computed a therapeutic index by the ratio of the median lethal dose and median effective dose method *in vivo* [35].

List of Abbreviations Used

4R6G6: The peptide with the formula $(RGRGRGRGRGRG)_4K_2K-NH_2$
ABR: antibiotic resistance
AMP: antimicrobial peptide
AUC: area under curve
CCA: Council of Canadian Academies
CL: alearance
ESI-MS: electrospray ionization mass spectrometry
H-NMR: proton nuclear magnetic resonance
HPLC: high performance liquid chromatography
K_e: elimination rate constant
LC-MS: liquid chromatography mass spectrometry
M/Z: mass to charge ratio
MIC: minimal inhibitory concentration
MRT: mean retention time

MS: mass spectrometry
PEI-*g*-PLL: poly(L-lysine) grafted to branched polyethylenamine
rpm: rotations per minute
SNAPP: structurally nanoengineered antimicrobial polypeptide polymer
TFA: trifluoroacetic acid
T_{half}: half life of elimination
V_d: volume of distribution

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent
As this is a research protocol which has yet to be conducted, it did not require ethics approval and/or patient consent.

Authors' Contributions
MS: Significantly contributed to study design, drafting and revising the manuscript, and gave final approval of the work to be published.
RA: Significantly contributed to study design, drafting and revising the manuscript, and gave final approval of the work to be published.
ES: Significantly contributed to drafting and revising the manuscript, and gave final approval of the work to be published.

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