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Novel Antimicrobial Peptide Fluoroquinolone Conjugates

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Introduction

Antimicrobial peptides (AMPs) have become of extreme interest over recent decades due to their natural origins, fluidity of amino acid sequences and low promotion of bacterial resistance pathways [1,2]. Unfortunately, these properties, particularly the low levels of bacterial resistance, are not wide spread in small molecule classes of antimicrobial agents, such as fluoroquinolones (FQs) [3]. Research into whether AMPs could enhance small molecule antibiotic agents has recently started to be investigated through either conjugation of small molecule agents or combination studies seeking synergistic properties [4-9]. However, these studies are limited in number and scope as many only provide a single conjugate of FQs to an AMP/amino acid candidate or produce combined dose regimes, with no covalent modifications. In addition, these studies generally have small sample sizes and results, such as MIC values, are varied. Building on this work, a novel fluoroquinolone-AMP conjugate consisting of several repeating units of ciprofloxacin has been produced. The rationale of this approach is to use the cationic charge and hydrophobic properties of ciprofloxacin, two essential traits of the AMPs' amphiphilic topology, while also keeping some of its original antibacterial activity. The candidates will be tested against gram-positive and gram-negative bacteria to assess their activity, as well as further studies to reveal mechanistic action of the peptide. They will aim in particular to evaluate if activity within the ciprofloxacin remains, once part of a larger AMP structure, or whether any advantages on conjugating FQs to AMPS, such as higher salt concentration tolerance or lower proteolytic susceptibility previously published about [4], are also present in these fluoroquinolone-AMP candidates.

Results and Discussion

To test this hypothesis a simple peptide sequence was designed to minimally retain two units contributing to the amphiphilic topologies and net cationic charge AMPs generally possess. As can be seen in Figure 1 the Cip residue is bound through the carboxyl group to the lysine ε-amino group. Arginine was chosen as a cationic unit while Cip was chosen as a cationic and hydrophobic unit, both contributing to the overall net charge. Ciprofloxacin was bound through the carboxyl group as this method of conjugations is well documented in the literature for FQs [4,6,9,10] so would help expedite the formation of initial candidates. A decamer with sequence H-[Lys(Cip)Arg]$_5$-NH$_2$ (**1**) was successfully synthesised. However, a significant byproduct was also detected. Coupling of the Cip unit to the free amine of a resin-bound peptide was difficult due to the length of the peptide chain causing steric bulk for amino acids close to the *C*-terminus and the inactivity of the carboxyl group owed to conjugation within the bicyclic ring. This difficulty required multiple coupling procedures with a particularly active coupling reagent, in this case HATU. Unfortunately, this uronium reagent can also react with the free amine of the lysine if reaction of the carboxyl group is not kinetically favourable. This leads to a guanylation to a lysine's ε-amino group which can been seen in Figure 1 producing **2 [11]**. This impurity could not be separated out via HPLC completely, therefore for initial susceptibility testing a combination of **1** and **2** was evaluated against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. Minimum Inhibitory Concentration (MIC) for these strains were higher than the highest concentration tested (256 μg/mL). This suggested that the amphiphilic topology of an AMP was not being met and the net charge of these peptides were too high. Interestingly, the parent peptide consisting of H(ArgTrp)$_5$NH$_2$ (**3**) also had a relatively high MIC against the same strains of *S. aureus* and *E. coli* with concentrations of 16μg/mL and 32-64μg/mL respectively. A new candidate was then hypothesised to increase hydrophobicity and include more varied residues, as in the sequence H-Lys(Cip)IleArgLys(Cip)ValArgLys(Cip)IleArg-NH$_2$ (**4**). Complete sequence assembly was performed to yield the resin-bound Fmoc-Lys(Mtt)IleArg(Pbf)Lys(Mtt)ValArg(Pbf)Lys(Mtt)Ile-Arg(Pbf)-Resin (**5**). Coupling of the Cip residue via HATU through the free ε-amino group was attempted by manual solid phase synthesis. Again, due to the aforementioned challenges with coupling

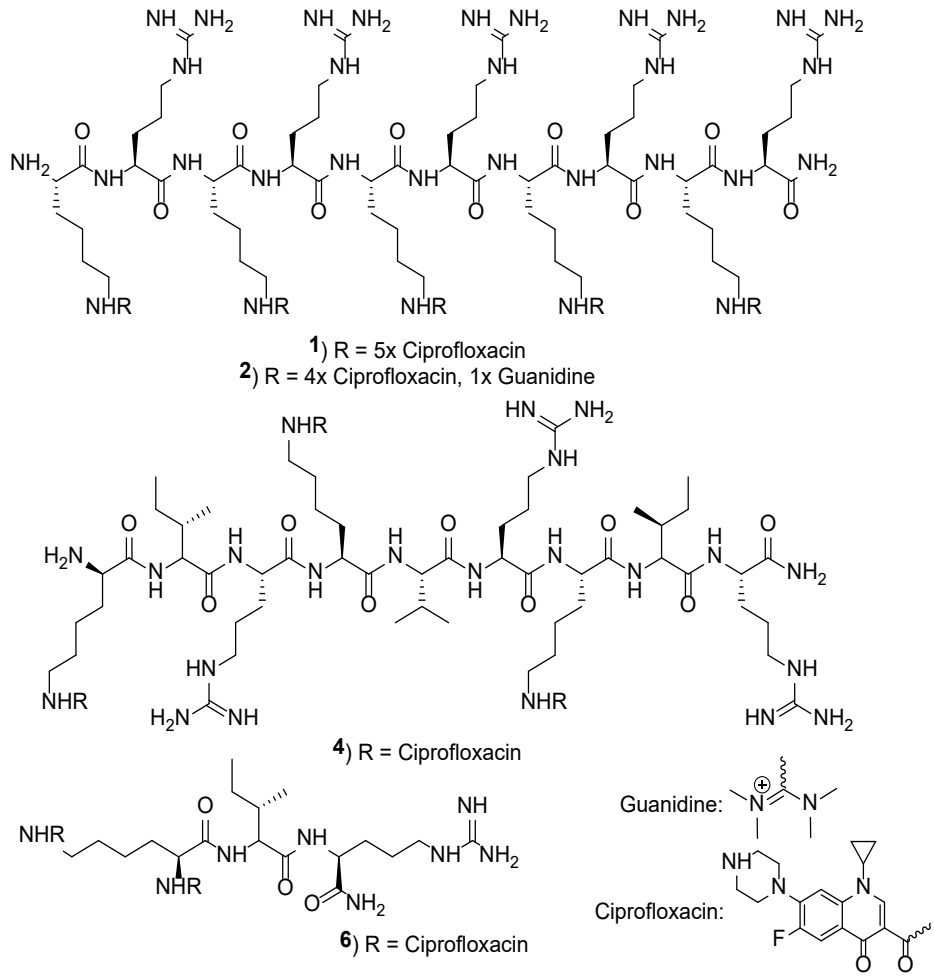

Fig. 1. Chemical structure of AMP-FQ candidates Top) ***1/2****, middle)* ***4*** *and bottom)* ***5*** *with corresponding side chains present during synthesis.*

as well as the increased steric hindrance of the branched residues, isoleucine and valine, only single and double Cip units were present, with no fully conjugated three Cip unit peptide detected in Figure 2. Several different methods were trialled to address this issue. Firstly, preactivation of the carboxyl group via an N-hydroxysuccinimide (NHS) ester was pursued. Although several attempts were made, no product was detected for the NHS ester of either BOC or FMOC protected Ciprofloxacin. Conventional coupling reagents were then pursued including HATU and PyBrop. In addition to coupling reagents, Ciprofloxacin was also converted into the acyl chloride *in situ* and then reacted with the free ε-amino group. After multiple couplings via each method, no amidation of the latter group was observed for all three sites. A new synthetic pathway was then devised to produce the 9-mer sequence stepwise, with the first three amino acids being coupled forming a tripeptide with lysine at the *N* terminus. The Cip unit would then be conjugated to the terminal lysine R group and upon successful coupling the peptide backbone would be extended until the next lysine were the Cip coupling would be repeated. This would produce the nonamer in three tripeptide steps and ensure the peptide chain causes minimal steric hindrance as the free ε-amino would always be on the *N* terminus.

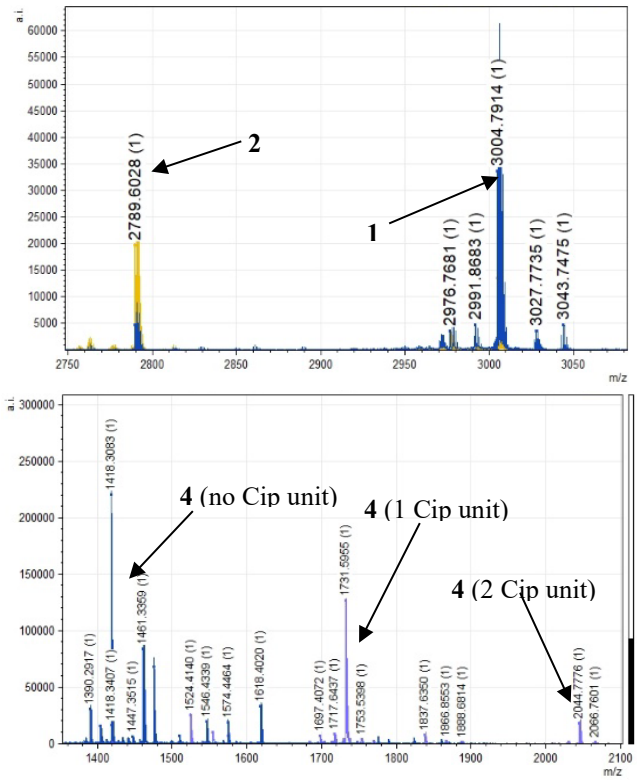

*Fig. 2: MALDI-TOF positive ion spectra containing derivatives of top) **3** and bottom) **4** and **5**.*

As a proof of concept, the tripeptide H-Lys(Mtt)IleArg(Pbf)-Resin bound (**6**) was synthesised and coupled to Cip. Preliminary results seem encouraging with conjugates containing a single Cip unit and double Cip unit (due to an unwanted loss of the *N*-terminal Fmoc group, caused by the automated synthesis) on the ε and α-amino groups. However, the by-product containing one Cip unit and one guanidine unit was also identified when using HATU.

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