7.0 Introduction

CAMPs interact with the cell membrane of the susceptible microorganisms to either accumulate in the membrane to cause increased permeability and loss of barrier function or enter the membrane to access the cytoplasmic targets [1]. Most of the natural CAMPs are large, have low potency and show toxicity to host cells [2,3]. Moreover, due to their peptidic nature, they suffer from poor bioavailability and poor proteolytic stability [4]. These features have significantly hampered their pharmaceutical development as therapeutic agents. Short designer CAMPs with increased half-lives offer excellent templates for future antibiotic drug design [5-7]. Different approaches are being tried towards increasing the effectiveness of CAMPs, alteration of sequences, inclusion of unnatural or D-amino acids or beta amino acids, cyclization of peptides, peptoid mimics, and synthesis of multivalent constructs of short peptides [8-10]. Short designer CAMPs that are less likely to induce resistance and minimize damage to host cells or tissues appear to be the most promising candidates. These CAMPs are generally synthesized through chemical reactions and the physical properties of these CAMPs depend on the method of preparation. Since these peptides have a wide range of clinical applications, appropriate toxicological screening is required prior to their use. Blood compatibility is the major problem in development of biocompatible drugs [11]. Hence, several techniques have been employed to determine the biocompatibility of peptides. In this chapter, the determination of biocompatibility/safety properties of the chosen peptides using standard methods is discussed.
7.1 Methodology

7.1.1 Mammalian cell cytotoxicity

The cytotoxicity of the AMPs (VSL2 & VS2) was determined using MTT assay against L6 cells at a cell density of $2 \times 10^4$ per well. These cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% fetal bovine serum in 96-well microtitre plate. After 24 hours of incubation, peptides (VSL2 & VS2 prepared in DMEM) at varying concentrations were added to cells and incubated for 24 hours at 37°C. After incubation, 20µL of MTT solution (5mg/ML) in PBS was added and incubated for 3 hours at 37°C to allow the formation of formazan product by MTT reduction. Supernatant was removed and DMSO was added to dissolve the crystals to obtain the purple coloured product. 1% Triton X-100 was used as positive control and untreated cells served as negative controls. The O.D at 570 and 690nm were used to calculate the percentage of viability [12].

\[
\text{% of cell viability} = \left( \frac{\text{O.D of drug treated sample}}{\text{O.D of untreated sample}} \right) \times 100
\]

7.1.2 Hemolytic assay

Blood from healthy volunteers was collected in heparinised vacutainers (IEC approval No: IEC-NI/09/DEC/13/37). Red blood cells (RBCs) were harvested by spinning at 1000 rpm for 10 min and were washed thrice with phosphate buffer saline (PBS). The packed RBCs were suspended in PBS to obtain 1% RBC. 100µL of 1% RBC was transferred to a tube and mixed with the desired concentrations of peptides. The tubes were incubated at 37°C for 1 hour and centrifuged at 1000 rpm for 10 minutes. The supernatant (100µL) was collected and O.D was read at 545 nm using a microtitre plate reader to detect the RBC lysis. RBCs lysed using 0.1% Triton X-100 showing
100% lysis, served as positive controls and cells incubated with PBS alone served as negative controls [13].

\[
\text{% of Hemolysis} = \frac{\text{O.D of Test} - \text{O.D of negative control}}{\text{O.D of positive control} - \text{O.D of negative control}} \times 100
\]

7.1.3 Platelet aggregation assay:

Human blood samples from healthy volunteers were obtained in vacutainers containing anticoagulant sodium citrate (IEC approval No: IEC-NI/09/DEC/13/37). The citrated blood was centrifuged at 1000 rpm for 10 minutes and the supernatant was again centrifuged at 4000 rpm for 10 minutes to get platelet rich plasma (PRP). 100µL of PRP was treated with peptides (VSL2 & VS2) of varying concentrations (10, 50 & 100µM). Aggregating property of platelets was observed microscopically. Collagen served as positive control [14].

7.2 Results

7.2.1 Mammalian cell cytotoxicity

The MTT assay is a good indicator of cell viability. This assay is based on the reduction of the MTT by those cells that remain viable after incubation with a test drug. MTT assay showed that both peptides are biocompatible and did not cause any toxicity to L6 fibroblast cells at the end of 24 hours of incubation. Viability of more than 80% was observed at 5µM concentration. It was also observed that peptides, VSL2 & VS2 above 5 x MIC values (50µM) showed some degree of toxicity to cells. However, the peptides provided the right balance between antimicrobial activity and cytotoxicity and were chosen for further studies (figure: 7.1).
Figure: 7.1 Mammalian-cell cytotoxicity (MTT assay) of peptide at MIC against L6 cell lines.

7.2.2 Hemolytic assay

Hemolytic assay was carried out to determine the hemocompatibility of peptides on RBCs. VSL2 was 12% hemolytic and VS2 was 3% hemolytic. Both the peptides, VSL2 & VS2 were not hemolytic. These results indicated that the peptides provided the right balance between cytotoxicity and hemolytic activity. The percent hemolysis of CAMPs is shown in figure: 7.2 (a) and figure 7.2 (b) shows the morphology of the exposed RBCs to CAMPs.
Figure: 7.2 (a) Percent hemolysis caused by CAMPs

![Percent hemolysis caused by CAMPs](image)

Figure: 7.2 (b) Morphology of RBCs exposed to CAMPs

![Morphology of RBCs](image)
7.2.3 Platelet aggregation assay

Platelet aggregation assay revealed that the AMPs did not cause aggregation of platelets even at the concentration of 100µM, (figure: 7.3) when compared to the control samples treated with collagen. Hence aggregating property of AMPs were verified and proved to be biocompatible.

Figure: 7.3 Platelet aggregation assay
7.3 Discussion

Cationic antimicrobial peptides (CAMPs) have become important candidates as potential therapeutic agents. Although the exact mode of action of CAMPs has not been established, it is generally accepted that the cytoplasmic membrane is the main target of many CAMPs. Peptide accumulation in the membrane causes increased permeability and a loss of barrier function, resulting in leakage of cytoplasmic components and cell death \[15,16\]. However, the major barrier for the use of AMPs as antibiotics is their toxicity or ability to lyse eukaryotic cells, normally expressed as hemolytic activity (toxicity to human red blood cells). This is the main hindrance with regard to their use as injectable therapeutics. The results of the hemolytic assay showed that the peptides were not hemolytic at low concentration and are therefore, hemocompatible. Platelets essentially contribute to the maintenance of vascular integrity and control of hemorrhage after injury. They can be activated by a variety of physiological factors and pharmacological agents \[17\] and hence the aggregating property is essential to determine the biocompatibility of a drug. The results of the aggregating assay showed that the AMPs did not cause aggregation of platelets and are therefore, biocompatible.

7.4 Conclusion

In the present chapter, the biocompatibility of CAMPs was studied and the results showed that the peptides were not toxic to L6 murine fibroblast cells at their MICs (10µM). The peptides did not cause hemolysis (10µM). They also did not cause aggregation of platelets which was confirmed by platelet aggregation assay.
7.5 References


