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Milad Mohkam, Navid Nezafat, Younes Ghasemi

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An in silico approach to de novo design of anti-microbial peptide from inspirited Komodo dragon's original VK6 peptide

Milad Mohkam

Allergy Research Center,
Shiraz University of Medical Sciences,
Shiraz, Iran
Email: miladmohkam@gmail.com

Navid Nezafat

Pharmaceutical Science Research Center,
Shiraz University of Medical Sciences,
Shiraz, Iran
Email: navidnezafat@yahoo.com

Younes Ghasemi*

Pharmaceutical Science Research Center,
Shiraz University of Medical Sciences,
P.O. Box 71345-1583, Shiraz, Iran
and
Department of Pharmaceutical Biotechnology,
School of Pharmacy,
Shiraz University of Medical Sciences,
Shiraz, Iran
Email: ghasemiy@sums.ac.ir
*Corresponding author

Abstract: Antimicrobial peptides (AMPs) function as the foremost barrier alongside fungi, bacteria, and viruses, thereby playing a pivotal role in innate immunity. These small peptides, ranging in size from 10 to 60 amino acid residues, are generated by various organisms. Reptiles, which are classified as ancient amniotes and have a wide range of ecological niches, are considered a valuable source of antimicrobial peptides (AMPs). In this study, we designed seven new AMPs to evaluate the impact of substituting tryptophan, phenylalanine, lysine, and arginine for enhancing antimicrobial activity using bioinformatic approaches. We assessed the relevant physicochemical traits using ProtParam and APD3 tools, and performed evaluations for possible allergenicity, antigenicity, and anti-inflammatory activity. The findings indicate that substitution of threonine, alanine, and valine amino acids in AMPs with tryptophan, phenylalanine, lysine, and arginine resulted in a noteworthy enhancement of the antimicrobial efficacy of the peptides designed, as compared to the original VK6 of Komodo dragon AMPs, accompanied by improved physicochemical properties. These findings highlight the

applicability of bioinformatic tools in designing and optimising novel AMPs with increased antimicrobial activity, which could be a promising approach in combating multi-drug resistant bacteria.

Keywords: bioinformatics; in silico; Komodo dragon; antimicrobial peptides; AMPs.

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Biographical notes: Milad Mohkam is an Assistant Professor of Pharmaceutical Biotechnology at the Allergy Research Center, Shiraz University of Medical Sciences. He holds an MSc in Microbiology and a PhD in Pharmaceutical Biotechnology, showcasing his dedication to academic excellence. His research interests encompass the diverse domains of biotechnology, microbiology, and bioinformatic studies. He has made contributions to the field, including his book 'Bacilli and Agrobiotechnology'.

Navid Nezafat received his PhD in Molecular Genetics in 2014, and his Postdoctoral degree in Pharmaceutical Biotechnology from Pharmaceutical Sciences Research, Shiraz University of Medical Sciences in 2017. He is an Assistant Professor at Pharmaceutical Sciences Research, Shiraz for more than six years. He published more than 120 papers in the fields of vaccine design, and protein engineering. His fields of interest are industrial biotechnology, bioinformatics, systems biology, and immunoinformatics.

Younes Ghasemi is the Department Leader of Pharmaceutical Biotechnology at Shiraz University of Medical Sciences (SUMS), and also the Head of Pharmaceutical Sciences Research Center at SUMS. He has been awarded as the Top Researcher at SUMS for four times. Currently, he has a record of more than 395 publications in peer-reviewed journals in the fields of recombinant proteins, enzyme technology, and molecular biotechnology. His more recent biomedical research focus is on the downstream processing, in silico biotechnology, and recombinant therapeutic proteins.

1 Introduction

The rise in prevalence of MDR infections has stimulated the exploration of novel approaches to combat microbes. These approaches include the identification of new antibiotics and the use of antimicrobial peptides (AMPs) (Hincapié et al., 2018). Cationic antimicrobial peptides (CAMPs) are being considered as a viable substitute for traditional antibiotics due to their strong anti-infective properties (Porto et al., 2018). The precise mechanism by which AMPs exert their effects is not yet fully understood. However, it is widely accepted that these peptides primarily target bacterial cell membranes and intracellular molecule (Buccini et al., 2020). Additionally, some AMPs are capable of possessing both antimicrobial and anti-biofilm activities, which may be driven by independent mechanisms within the same peptide (Cao et al., 2020). AMPs are believed to pose a challenge to bacterial resistance development due to their ability to swiftly eliminate bacteria by targeting either the bacterial cell membrane or intricate mechanisms

within the bacterial cell (Magana et al., 2020). AMPs are recognised for their wide-ranging activities, as peptides that are active have demonstrated effectiveness against both gram-positive and gram-negative bacteria, fungi, and typically membrane-bound viruses (Huan et al., 2020). In addition, it has been observed that AMPs have the ability to directly regulate the immune response of the host. This characteristic is believed to be a crucial factor in their in-vivo functionality (Boparai and Sharma, 2020).

Bacterial infections, including *Acinetobacter baumannii* and *Pseudomonas aeruginosa* infections, pose significant challenges, particularly in hospital settings. *A. baumannii* is a pathogenic gram-negative bacterium that is notorious for causing severe nosocomial infections, producing biofilms, and exhibiting resistance to the majority of antibiotics (Ayoub Moubareck and Hammoudi Halat, 2020). Given that this bacterium is commonly associated with infections in immunocompromised patients, the increasing incidence of community-acquired infections with multidrug-resistant (MDR) *A. baumannii* has attracted much attention (Ayoub Moubareck and Hammoudi Halat, 2020). Currently, the number of antibacterial agents with reliable anti-pseudomonal activity is limited to a small number of agents in three major antibiotic categories: aminoglycosides, lactams, and fluoroquinolones (Rose et al., 2020). The first-line treatment for *A. baumannii* infections currently entails prescription of a carbapenem or imipenem, but resistance to carbapenems is becoming increasingly common (Bassetti et al., 2021). Other treatment options include aminoglycosides, tigecycline, and polymyxins (Bassetti et al., 2021). However, the MDR Acinetobacter isolation has increased from 6.7% to 29.9% between 1993 and 2004, underscoring the need for better and novel therapies (Morris and Cerceo, 2020).

The utilisation of animals as a potential reservoir of AMPs is a highly auspicious avenue, given their robust immune systems that have evolved to combat microorganisms (Wang et al., 2019). The *Varanus komodoensis*, commonly known as the Komodo dragon, along with other reptilian species, are considered to be archaic animals in terms of their evolutionary history. It has been demonstrated in prior research that leukocyte and plasma extracts derived from these animals exhibit notable antimicrobial properties (Bishop et al., 2017). The Komodo dragon employs a hunting technique that involves administering a bite to its prey, leading to the eventual demise of the prey. This is attributed to the presence of approximately 57 pathogenic bacterial species residing in the dragon's saliva and oral cavities, which ultimately causes infection (D'Amore et al., 2011). The remarkable capacity of Komodo dragons to withstand the presence of numerous pathogenic bacterial strains in their saliva, while remaining asymptomatic, underscores the inherent advantages of their innate immune system and merits additional scrutiny. The present study endeavours to employ computational tools to design novel AMPs sourced from the AMP library of the Komodo dragon, with the objective of combating MDR *A. baumannii*. Our objective is to investigate the prospective utility of these peptides as a novel reservoir of agents in the battle against MDR *A. baumannii*.

2 Methods

2.1 Sequence retrieval

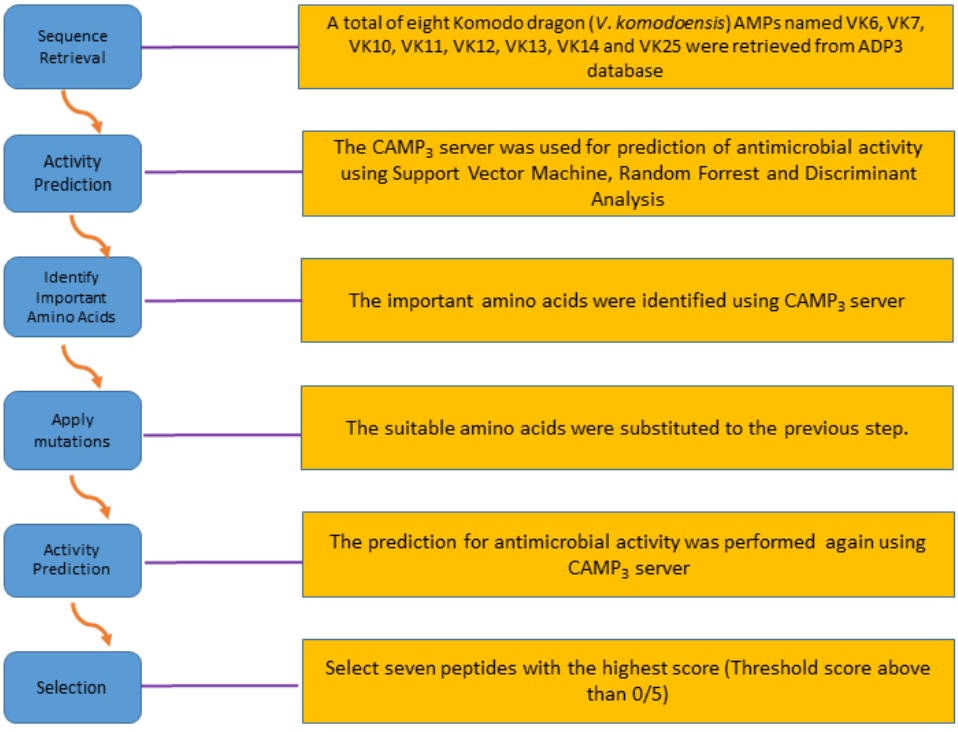
The AMP sequences of the Komodo dragon were procured from the APD3 database (http://aps.unmc.edu/AP/main.php), which is widely recognised as a reliable repository

for both synthetic and natural AMPs. The utilisation of the database not only facilitates the retrieval of extant sequences, but also affords the opportunity to design novel AMPs and prognosticate their attributes (Wang et al., 2016).

2.2 De novo anti-microbial peptide designing

This study utilised template-assisted approaches and sequence modification strategies to design amphipathic random coil AMPs. To increase the peptides' positive net charge, threonine (T), alanine (A), and valine (V) were substituted with arginine (R) and lysine (L). Tryptophan (W) and phenylalanine (F) were also incorporated to increase the peptides' amphipathicity, allowing for greater interaction with the lipid bilayer and aqueous environment, which ultimately enhances antimicrobial activity. Additionally, glycine (G) was added to the N-terminal end to boost peptide activity, while proline (P) was included to prevent α-helix formation (Figure 1).

Figure 1 Strategies performed in order to design enhanced novel antimicrobial from Komodo dragon AMPs (see online version for colours)



2.3 Prediction different activities of AMP

The assessment of the antimicrobial efficacy of individual amino acid substitutions was conducted through various web-based resources. These included CAMPR3 (http://www.camp.bicnirrh.res.in/predict/), which uses discriminant analysis (DA), support vector machine (SVM), and random forest (RF) algorithms to predict

antimicrobial activity (Waghu and Idicula-Thomas, 2020), DBAASP (https://dbaasp.org/prediction/special) (Pirtskhalava et al., 2021). iAMPpred (http://cabgrid.res.in:8080/amppred/), which uses a SVM method to evaluate antifungal activity based on physicochemical and compositional traits as well as structural properties (e.g., peptide characteristics); Meta-iAVP (http://codes.bio/meta-iavp/), which was used to assess antiviral activity (Schaduangrat et al., 2019). AtbPpred (http://thegleelab.org/AtbPpred/) (Manavalan et al., 2019), which utilises a two-layer machine learning (ML)-based predictor to evaluate anti-tuberculosis peptides and has an accuracy of 88.3%; and dPABBs (http://ab-openlab.csir.res.in/abp/antibiofilm/), which predicts anti-biofilm peptides using six SVM and Weka models with a specificity, sensitivity, accuracy, and Matthews correlation coefficient (MCC) of 97.73%, 92.50%, 95.24%, and 0.91, respectively (Sharma et al., 2016). In all experiments, the score above the 0.5 was considered as positive value.

2.4 Prediction other important biological activities of AMP

The AntiCP 2.0 server was used to conduct an in-silico analysis of the peptide's anti-cancer activity; this analysis produced the highest MCC of 0.80 and area under the receiver operating characteristic (AUROC) of 0.97 in the training dataset. (https://webs.iiitd.edu.in/raghava/anticp2/predict.php) (Agrawal et al., 2021). To predict bioactive peptides, the Peptide Ranker server was employed, which utilises a novel N-to-1 neural network (http://distilldeep.ucd.ie/PeptideRanker/). Two servers, C2Pred (http://lin-group.cn/server/C2Pred) and MLCPP (http://www.thegleelab.org/MLCPP/), were used to forecast the cell penetration potential of AMPs. C2Pred uses a SVM with an accuracy rate of 83.6% (Tang et al., 2016), while MLCPP employs four different ML approaches with an accuracy rate of 89.6% (Manavalan et al., 2018b). The HLP server (https://webs.iiitd.edu.in/raghava/hlp/interactive.htm) was employed to predict the half-life of peptides in an intestine-like environment (Sharma et al., 2014). The AnOxPePred – 1.0 server, which employs a deep learning algorithm, was used to assess the antioxidant activity (https://services.healthtech.dtu.dk/service.php?AnOxPePred-1.0) (Olsen et al., 2020).

2.5 Predicting physicochemical properties, allergenicity, antigenicity and anti-inflammatory of peptide

The physicochemical traits of Komodo Dragon's peptides as well as the designed AMPs were evaluated using APD3 tools (https://aps.unmc.edu/tools) and ProtParam tool (https://web.expasy.org/protparam) (Wang et al., 2016, Gasteiger et al., 2005).

The allergenic potential of the designed AMPs was assessed using three distinct tools. AllerCatPro (https://allercatpro.bii.a-star.edu.sg/) achieved an overall accuracy of 84% (Maurer-Stroh et al., 2019), whereas AllerTOP v. 2.0 (https://www.ddg-pharmfac.net/AllerTOP/method.html) employed auto cross covariance (ACC) transformation of protein sequences into uniform equal-length vectors and attained an accuracy rate of 85.3% (Dimitrov et al., 2014). AlgPred (https://webs.iiitd.edu.in/raghava/algpred2/batch.html) achieved a MCC of 0.85 (Sharma et al., 2021). Antigenicity of AMPs was evaluated by two servers, predicting antigenic peptides with an accuracy rate of 75% (http://imed.med.ucm.es/Tools/antigenic.pl) and SVMTriP which utilised a SVM, resulting in a sensitivity of 80.1% and a precision of 55.2% (Yao

et al., 2012). Furthermore, in silico anti-inflammatory activity of the designed peptide was evaluated via the AIPpred server (http://thegleelab.org/AIPpred/index.html) (Manavalan et al., 2018a).

2.6 3D structure prediction and evaluation of modelled peptide

To predict the 3D structures of the designed AMPs, we utilised the PEP-FOLD 3.5 server (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3) and selected the top five models with the highest scores (Thévenet et al., 2012). AMPs that had higher overall parameters, such as higher APM activity scores and nontoxicity to Erythrocytes, were further evaluated. Among these AMPs, VK6 peptide was selected due to its unique structure, a random coil, as opposed to the typical α-helix and β-sheet structures of AMPs. Additionally, we selected indolicidin, a bovine-derived AMP, as a random coil AMP for comparison purposes.

2.7 α helix formation and functional analysis of peptide

To determine the presence of putative amphipathic helices in the amino acid sequence of the designed peptides, an in silico analysis was conducted using the HELIQUEST server (http://heliquest.ipmc.cnrs.fr/) (Gautier et al., 2008).

Furthermore, the likelihood of membrane-spanning regions or extracellular nature of the peptides was predicted using the TMHMM tool, which utilises a hidden Markov model with an accuracy rate of 87% (www.cbs.dtu.dk/services/TMHMM) (Krogh et al., 2001).

2.8 Predicting the aggregation propensity of AMPs

The amyloidogenic or aggregation propensities of the designed AMPs were assessed using two online prediction tools. PASTA, which has a true positive prediction rate of 80% (http://old.protein.bio.unipd.it/pasta2/#) (Walsh et al., 2014), and AGGRESCAN, which calculates aggregation-propensity values per amino acid (http://bioinf.uab.es/aggrescan/) were employed (Conchillo-Solé et al., 2007).

3 Results

3.1 Sequence retrieval and de novo designing of AMP

A total of eight Komodo dragon (*V. komodoensis*) AMPs named VK6, VK7, VK10, VK11, VK12, VK13, VK14 and VK25 were retrieved from ADP3 database (Table 1). Initial study showed that only VK6 and VK25 had random coiled structure in comparison to other AMPs (data not shown). In this regard, the VK6 was opted as a template for *de novo* designing of AMPs. Furthermore, the indolicidin was selected as a random coil AMP for comparison. A number of AMPs were designed according to the VK6 peptide. For increasing positive net charge, the valine, alanine and threonine were replaced by arginine and lysine. In addition, tryptophan was also added in order to increase the amphipathicity. Other amino acid substitutions were also determined.

Table 1 The results of AMPs score obtained from CAMP3 server.

Peptide sequence (name/substitution)	*CAMPs3 online server*		
	SVM	*RF*	*DA*
VK6 AVKPKTAKPKTAKPKTA	0.996	0.7195	0.993
MM1 GFKPKWPKPKIKKPKIK	1.000	0.7795	1.000
MM2 WFRPKWPKPRIKKPRIK	0.999	0.764	0.996
MM3 WFRPKWPKPRIPKPRIK	0.999	0.8255	0.993
MM4 GFRPKWPKPRIPKPRIK	0.998	0.82	0.997
MM5 GFRPKWPKPRIPKWRIK	0.999	0.8125	0.992
MM6 GFRPKWPKPRIPKPWIK	0.999	0.823	0.978
MM7 GFRWKWFKFRWWKPWIK	1.000	0.98	1.000
Indolicidin ILPWKWPWWPWRR	0.999	0.991	0.997

Notes: SVM: surface vector machine, RF: random forest, DA: discriminant analysis.
The score above than 0.5 was considered as positive value.

3.2 Prediction different activities of the designed AMP

In this study, more than 50 AMPs were designed and evaluated using the CAMP3 server to identify the most promising candidates (Table 1). Seven peptides with the highest scores were selected for further evaluation. Based on the preliminary findings, it was observed that the replacement of alanine, valine, and threonine with positively charged amino acids resulted in a theoretical enhancement of antimicrobial activity, which was found to be similar to that of the original VK6 and indolicidin. Similarly, substituting with Tryptophan and Phenylalanine showed a similar pattern. Table 2 displays the antimicrobial efficacy of the peptides against strains of bacteria that are obtained from the DBAASP database. In this regards, all of the designed AMPs were found to be non-hemolytic against erythrocytes, except for indolicidin. The anti-tuberculosis activity prediction from the AtbPpred online server is presented in Table 3, while Table 4 provides information on the predicted antifungal (using iAMPpred tools), antiviral (using Meta-iAVP tool), and anti-biofilm (using PABBs tool) activities. These analyses identified seven newly designed active peptides, all of which demonstrated anti-tuberculosis activity. While MM7 did not show any antifungal activity, the others exhibited a combination of antiviral, antifungal, anti-biofilm, or anti-tuberculosis activity. In total, three peptides showed a complete range of antimicrobial activities (antibacterial, antifungal, and antiviral), comparable to indolicidin.

Table 2 The prediction of antibacterial activity against some standard bacterial obtained from DBAASP database

	E. coli (*ATCC 25922*)		*P. aeruginosa* (*ATCC 27853*)		*k. pneumonia*		*S. aureus* (*ATCC 25923*)		*Human erythrocytes*		*B. subtilis*		*C. albicans*		*S. cerevisiae*	
	Class	*Predictive value (type)*	*Class*	*Predictive value (type)*	*Class*	*Predictive value (type)*	*Class*	*Predictive value (type)*	*Class*	*Predictive value (type)*	*Class*	*Predictive value (type)*	*Class*	*Predictive value (type)*	*Class*	*Predictive value (type)*
VK6	Active	0.53 (PPV)	Not active	0.69 (NPV)	Not active	0.95 (NPV)	Not active	0.76 (NPV)	Not active	0.95 (PPV)	Active	0.81 (PPV)	Not active	0.94 (NPV)	Not active	0.89 (NPV)
MM1	Active	0.51 (NPV)	Not active	0.64 (NPV)	Not active	1.00 (NPV)	Not active	0.69 (NPV)	Not active	0.96 (PPV)	Not active	0.76 (NPV)	Not active	0.89 (NPV)	Not active	0.75 (NPV)
MM2	Active	0.61 (PPV)	Not active	0.57 (NPV)	Not active	1.00 (NPV)	Not active	0.60 (NPV)	Not active	0.87 (PPV)	Not active	0.76 (NPV)	Not active	0.86 (NPV)	Not active	0.66 (NPV)
MM3	Active	0.65 (PPV)	Not active	0.66 (NPV)	Not active	1.00 (NPV)	Not active	0.62 (NPV)	Not active	0.91 (PPV)	Not active	0.76 (NPV)	Not active	0.88 (NPV)	Not active	0.70 (NPV)
MM4	Active	0.57 (PPV)	Not active	0.76 (NPV)	Not active	1.00 (NPV)	Not active	0.60 (NPV)	Not active	0.92 (PPV)	Not active	0.76 (NPV)	Not active	0.89 (NPV)	Not active	0.75 (NPV)
MM5	Active	0.65 (PPV)	Not active	0.73 (NPV)	Not active	1.00 (NPV)	Not active	0.63 (NPV)	Not active	0.94 (PPV)	Not active	0.76 (NPV)	Not active	0.87 (NPV)	Not active	0.69 (NPV)
MM6	Active	0.64 (PPV)	Not active	0.78 (NPV)	Not active	1.00 (NPV)	Not active	0.75 (NPV)	Not active	0.86 (PPV)	Not active	0.76 (NPV)	Not active	0.89 (NPV)	Not active	0.73 (NPV)
MM7	Active	0.81 (PPV)	Active	0.63 (PPV)	Not active	0.86 (NPV)	Active	0.73 (PPV)	Not active	0.64 (PPV)	Active	0.81 (PPV)	Active	0.76 (PPV)	Active	0.83 (PPV)
Indolicidin	Not active	0.51 (NPV)	Active	0.51 (PPV)	Not active	1.00 (NPV)	Active	0.56 (PPV)	Active	0.99 (NPV)	Not active	0.76 (NPV)	Active	0.76 (PPV)	Active	0.83 (PPV)

Notes: Active peptide implies MIC < 25 μg/ml. Non-active peptide implies MIC > 100 μg/ml. Positive predictive value (PPV) for peptides predicted as active and negative predictive value (NPV) for peptides predicted as not active. Taking into account, that for the evaluation activity against Human erythrocytes, non-active peptides are considered as positive, the server gives PPV for peptides not active against human erythrocytes, and NPV otherwise.

Table 3 The prediction of anti-tuberculosis using AtbPpred online server

	AtbP or non-AtbP	*Probability*
VK6	Non-AtbP	0.18214
MM1	AtbP	0.54643
MM2	AtbP	0.60714
MM3	AtbP	0.62857
MM4	AtbP	0.54286
MM5	AtbP	0.67143
MM6	AtbP	0.66786
MM7	AtbP	0.81786
Indolicidin	AtbP	0.89643

Note: The score above than 0.5 was considered as positive value.

Table 4 The prediction of antifungal, antiviral and anti-biofilm formation

	Antifungal peptide iAMPpred		*Antiviral peptide (Meta-iAVP)*		*Anti-biofilm prediction (dPABBs)*	
	Prediction	*Score*	*Prediction*	*Score*	*Prediction*	*Score*
VK6	Yes	0.80	No	0.112	No	0.33
MM1	Yes	0.60	No	0.11	Yes	0.76
MM2	Yes	0.64	Yes	0.736	Yes	0.81
MM3	Yes	0.65	Yes	0.854	Yes	0.81
MM4	Yes	0.84	No	0.044	Yes	0.81
MM5	Yes	0.68	Yes	0.954	Yes	0.84
MM6	Yes	0.64	Yes	0.964	Yes	0.84
MM7	No	0.37	Yes	0.984	Yes	0.84
Indolicidin	Yes	0.81	Yes	0.54	Yes	0.84

Note: The score above than 0.5 was considered as positive value.

3.3 Prediction other important biological activities of AMP

Various biological functions beyond antimicrobial activities, such as anticancer and antioxidant activities, bioactive peptide activity, intestinal half-life, and cell penetration activity were also evaluated (Tables 5 and 6). The anticancer activity predictions showed that all designed AMPs, except MM1, as well as the original VK6 and indolicidin, possessed acceptable anticancer activity. The prediction results for metal chelating activity (MCA) and free radical scavenging (FRS) revealed that MM3, MM4, and MM7 peptides had relatively high FRS scores (FRS threshold = 0.43–0.51) compared to the original VK6 (FRS = 0.33). However, indolicidin showed an extremely high FRS score. In contrast, no peptide reached the score threshold (MCA > 0.3) for MCA, including VK6 and indolicidin. The bioactive peptide prediction, run by the Peptide Ranker online tool, showed that all designed AMPs, along with indolicidin, had excellent bioactivity scores (> 0.8), which were relatively higher than VK6 peptide score (= 0.26). The HLP server was employed in predicting the half-life of the peptides that were designed

specifically for the intestinal environment. The findings indicate that, with the exception of MM7, all AMPs that were designed, including VK6, demonstrated a prolonged half-life within the intestinal environment, while indolicidin had a normal half-life. Furthermore, the cell penetration activity of the peptides was evaluated using the C2Pred and MLCPP online servers. The findings of the C2Pred server showed that only MM2, MM3, MM7, indolicidin, and VK6 had cell-penetrating activity, while the outcomes derived from the MLCPP server have demonstrated that the entirety of the peptides that were formulated possess the ability to penetrate cells. However, high uptake efficiency was only observed for MM2, MM3, MM5, MM7, indolicidin, and VK6, which was consistent with the findings of the C2Pred server.

Table 5 The results of anticancer, anti-oxidant activity, half-life peptides in intestinal environment and bioactive peptides

	Anticancer peptide		*AnOxPePred – 1.0*		*HLP server*		*Peptide ranker*
	Prediction	*Score*	*FRS score*	*Chelation score*	*Half life (sec)*	*Stability*	*Score*
VK6	No	0.129354	0.3330	0.1790	6.347	High	0.2684
MM1	No	0.204708	0.4254	0.1838	6.347	High	0.8969
MM2	Yes	0.994653	0.4937	0.1673	6.347	High	0.9200
MM3	Yes	0.976333	0.5224	0.1666	6.347	High	0.8943
MM4	Yes	0.836008	0.5179	0.1693	6.347	High	0.8989
MM5	Yes	0.962002	0.4771	0.1502	6.347	High	0.8618
MM6	Yes	0.587172	0.4889	0.1712	6.397	High	0.9335
MM7	Yes	0.500000	0.5116	0.1369	0.590	Normal	0.9888
Indolicidin	No	0.277905	0.6698	0.1548	0.594	Normal	0.9900

Note: The score above than 0.5 was considered as positive value except for chelating activity score, which score above than 0.13 consider as positive for activity.

Table 6 The prediction of cell penetration activity of designed AMPs from C2Pred and MLCPP servers

	C2Pred server		*MLCPP server*			
	Prediction	*Score*	*Prediction*	*Score*	*Uptake efficiency*	*Score*
VK6	Yes	0.893211	Yes	0.8281	Low	0.2148
VK6 MM1	No	0.474846	Yes	0.8849	Low	0.3005
VK6 MM2	Yes	0.865380	Yes	0.9545	High	0.6990
VK6 MM3	Yes	0.666219	Yes	0.9298	High	0.5898
VK6 MM4	No	0.288744	Yes	0.8984	Low	0.4273
VK6 MM5	No	0.365499	Yes	0.9355	High	0.5797
VK6 MM6	No	0.388440	Yes	0.8513	Low	0.4801
VK6 MM7	Yes	0.630262	Yes	0.8921	High	0.6970
Indolicidin	Yes	0.871117	Yes	0.8736	High	0.5436

Note: The score above than 0.5 was considered as positive value.

3.4 Evaluating physicochemical properties of AMP

The AMPs were tested for their physicochemical properties with the help of the ProtParam program, the Antimicrobial Peptide Database Calculator, and Predictor APD3. The hydrophobic ratio of the peptides varied from 24 (MM4) to 50 (indolicidin). The AMPs exhibited a range of net charges, spanning from +3 (indolicidin) to +8 (MM1 and MM2), with a corresponding range of isoelectric points (pI), spanning from 10.70 (VK6) to 12.32 (MM2). The total aliphatic index of the peptides ranged from 22.94 (MM7) to 60 (indolicidin), and the instability index varied from –35 (MM1) to 76 (indolicidin). The Boman index values ranged from 1.66 (MM1) to 3.23 (MM2) (Table 7).

Table 7 The prediction of physicochemical properties of designed AMPs

Peptide sequence (name/substitution)	*Hydrophobic ratio (%)*	*Boman index*	*GRAVY (kcal/mol)*	*Instability index*	*Aliphatic index*	*Total net charge*	*pI*	*Hydrophobicity <H>*	*Hydrophobic moment <μH>*
VK6 AVKPKTAKPKTAKPKTA	29	1.74	–1.112	–5.59	40.59	+6	10.70	–0.032	0.046
MM1 GFKPKWPKPKIKKPKIK	24	1.66	–1.594	–32.71	44.88	+8	10.85	0.153	0.344
MM2 WFRPKWPKPRIKKPRIK	29	3.23	–1.729	–6.99	45.88	+8	12.32	0.282	0.334
MM3 WFRPKWPKPRIPKPRIK	29	2.91	–1.594	–3.69	45.88	+7	12.31	0.382	0.263
MM4 GFRPKWPKPRIPKPRIK	24	2.99	–1.565	–3.69	45.88	+7	12.31	0.250	0.306
MM5 GFRPKWPKPRIPKWRIK	29	2.85	–1.524	5.18	45.88	+7	12.31	0.340	0.324
MM6 GFRPKWPKPRIPKPWIK	29	1.97	–1.353	–0.95	45.88	+6	12.03	0.442	0.498
MM7 GFRWKWFKFRWWKPWIK	41	1.71	–1.071	56.84	22.94	+6	12.03	0.774	0.465
Indolicidin ILPWKWPWWPWRR	50	1.06	–1.069	76.28	60	+3	12.01	1.069	0.207

3.5 Allergenicity, antigenicity and anti-inflammatory prediction

The peptides' allergenicity and antigenicity were assessed through a range of online servers, including AllerCatPro, AllerTOP v. 2.0, AlgPred, Antigenic Peptide Prediction, and SVMTriP, which all indicated no potential for allergenicity or antigenicity. Additionally, the AIPpred server was utilised to predict anti-inflammatory effects, which showed that all designed peptides possessed anti-inflammatory activity, with the exception of Vk6 and indolicidin, which did not follow the same pattern (Table 8).

Table 8 The prediction of allergenicity, antigenicity and anti-inflammatory features of designed AMPs

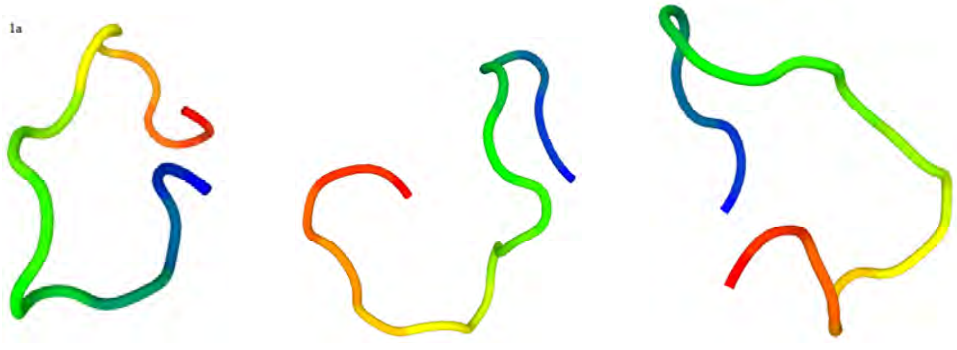
Alignment model	*AllerCatPro*	*AllerTOP v. 2.0*	*AlgPred*	*Antigenic peptide prediction*	*SVMTriP*	*AIPpred*	
						Prediction	*Score*
VK6	No	No	No	No	No	No	0.4605
MM1	No	No	No	No	No	Yes	0.6512
MM2	No	No	No	No	No	Yes	0.6000
MM3	No	No	No	No	No	Yes	0.5884
MM4	No	No	No	No	No	Yes	0.6023
MM5	No	No	No	No	No	Yes	0.6512
MM6	No	No	No	No	No	Yes	0.6395
MM7	No	No	No	No	No	Yes	0.6023
Indolicidin	No	No	No	No	No	No	0.4977

Note: The score above than 0.5 was considered as positive value.

3.6 Evaluating and predicting the three-dimensional shape of designed peptides

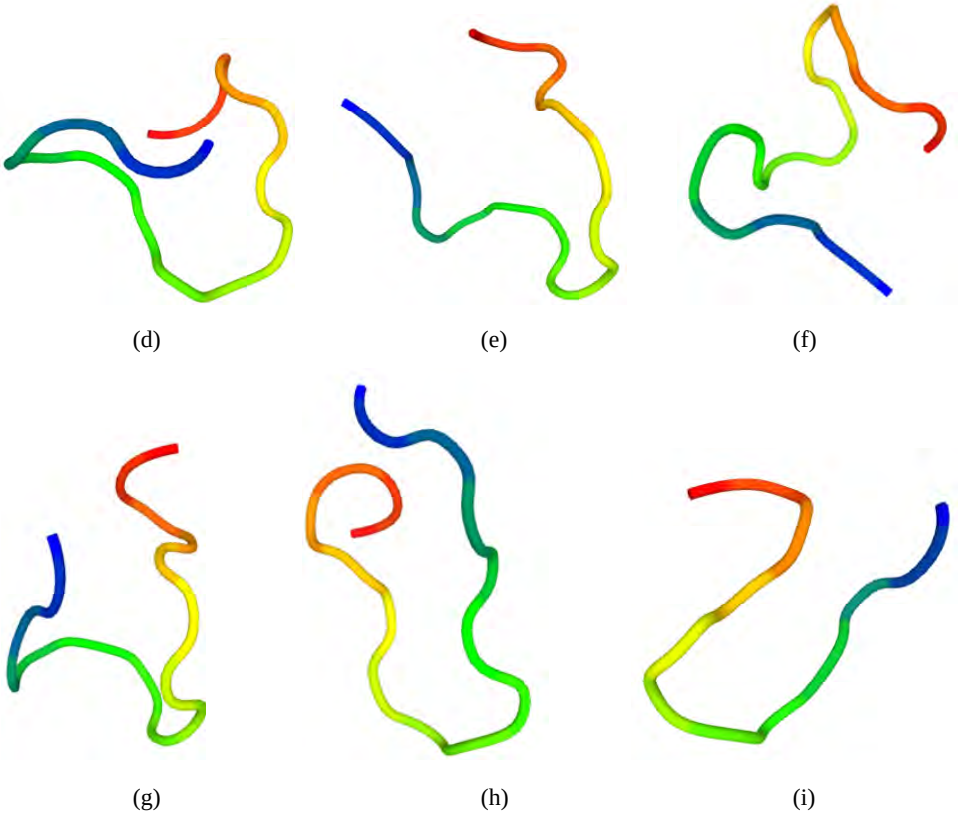
The 3D structures of the designed AMPs were predicted by deploying the PEP-FOLD 3 online server, as depicted in Figure 2. The quality of the models was evaluated based on the Qmean score, which exceeded 0.9, indicating their validity. The results showed that all of the synthesised AMPs possessed a random coil conformation, which closely resembled that of indolicidin.

Figure 2 3D structure prediction of designed peptides run by PepFold3 server, (a) original VK6, (b) MM1, (c) MM2, (d) MM3, (e) MM4, (f) MM5, (g) MM6, (h) MM7, (i) indolicidin (see online version for colours)



(a) (b) (c)

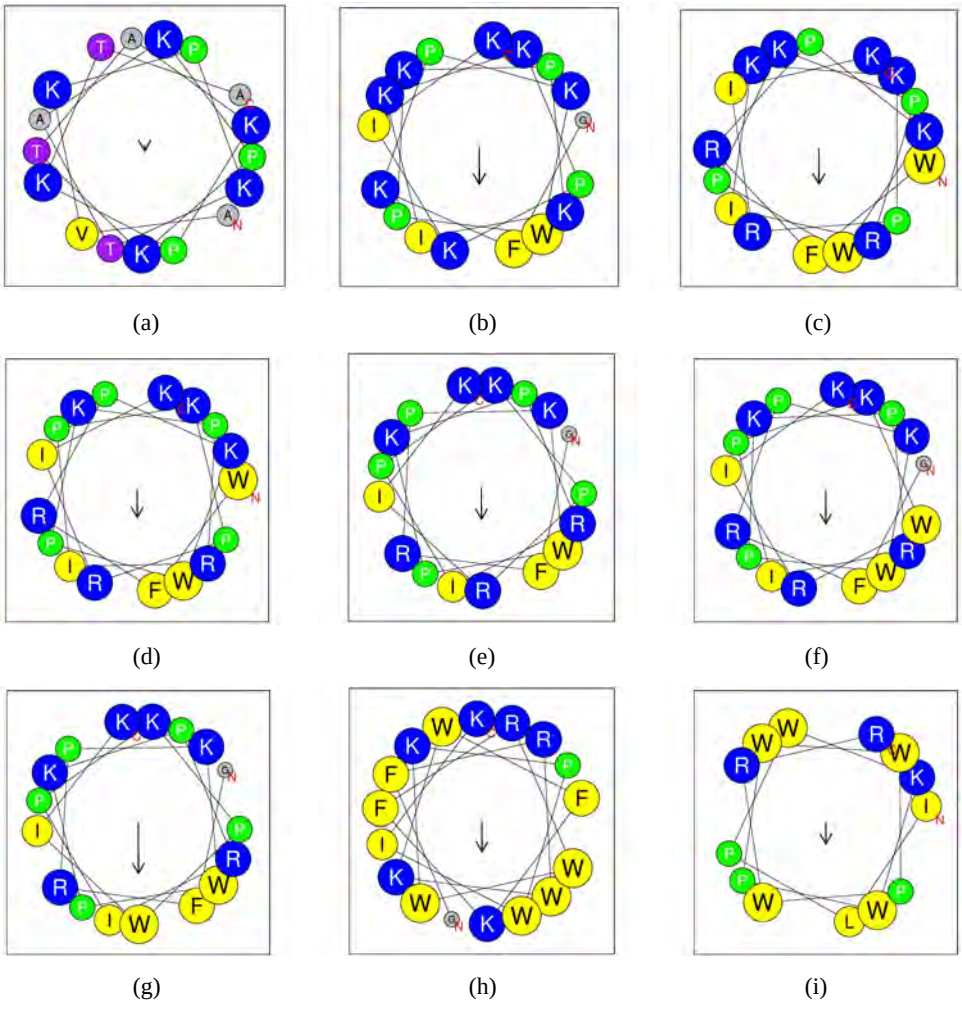
Figure 2 3D structure prediction of designed peptides run by PepFold3 server, (a) original VK6, (b) MM1, (c) MM2, (d) MM3, (e) MM4, (f) MM5, (g) MM6, (h) MM7, (i) indolicidin (continued) (see online version for colours)



3.7 Analysis of α helix formation, and prediction of the aggregation propensity of AMPs

The visualisation of amino acid distribution within the AMP structures was achieved through the utilisation of helical wheel projections, as demonstrated in Figure 3. Through the utilisation of the helical structures, it was observed that all of the designed AMPs exhibited amphipathic characteristics (Figure 3). Additionally, the distributions of hydrophilic and hydrophobic amino acids in the projections of the designed AMPs were similar. The primary difference among the designed AMPs lies in their hydrophobic and amphipathic properties, as well as the amount of amino acids with positive charges they encompass. Analysis using the online TMHMM server software indicated that the synthesised AMPs were incapable of traversing the membrane, and thus localised on the external surface of the membrane of bacterial cell. Moreover, the PASTA and AGGRESCAN servers were used to predict the likelihood of AMP aggregation, but no such possibility was detected.

Figure 3 Helical wheel projections of designed peptides forecasted by the HeliQuest online tool, (a) original VK6, (b) MM1, (c) MM2, (d) MM3, (e): MM4, (f) MM5, (g) MM6, (h) MM7, (i) indolicidin (see online version for colours)



Notes: Blue: basic residues; red: acidic residues; Yellow: hydrophobic residues; green: special residues; purple and pink: polar residues; arrow: hydrophobic moment.

4 Discussion

The advent of MDR strains, exemplified by *S. aureus*, *P. aeruginosa*, and *A. baumannii*, presents a formidable peril to the well-being of the populace, especially in medical facilities, where patients are especially susceptible. The escalation of *A. baumannii* as a perilous nosocomial pathogen, particularly in intensive care units, has undergone a significant surge in recent years (Bassetti et al., 2021). Consequently, the growing incidence of antibiotic resistance among *S. aureus*, *A. baumannii*, and *P. aeruginosa* underscores the pressing necessity for the creation of novel antibacterial agents.

AMPs exhibit promise as a substitute for conventional antibiotics due to their broad spectrum of activity against diverse organisms, such as mycobacteria, bacteria, viruses, fungi, and cancer cells. Additionally, they have lower toxicity towards eukaryotic cells (Hu et al., 2013). AMPs are capable of killing bacteria through a range of mechanisms, such as disrupting membranes, interfering with bacterial metabolism, and targeting intracellular constituents (Bechinger and Gorr, 2017, Van Hoek, 2014). Furthermore, the majority of AMPs have a cationic nature, resulting in an overall positive charge that enhances their electrostatic interaction with negatively charged bacterial membranes (Yeaman and Yount, 2003).

AMPs are produced by various living organisms, including the Komodo dragon. The Komodo dragon is a distinctive source of AMPs due to its capacity to survive in harsh environments and possess a highly developed immune system (van Hoek et al., 2019; Bishop et al., 2017). To date, a total of eight AMPs have been discerned within the salivary ducts of the Komodo dragon, which allow it to adapt to environments with a heavy microbial load (Bishop et al., 2017). In this study, we designed seven novel AMPs based on the original VK6 AMP from the Komodo dragon, which demonstrated enhanced activity compared to VK6 and contained amino acids comprising of Tryptophan, Phenylalanine, Glycine, and Isoleucine. These modifications led to an increase in overall positive charge, comparative hydrophobicity, and hydrophobicity, resulting in improved antimicrobial activity compared to VK6 and indolicidin. Our analysis suggests that the replacement of Tryptophan in the sequence of the designed AMPs, as well as the incorporation of Arginine or Lysine (to increase positive net charge), are responsible for the observed improvement in antimicrobial activity, as inferred from CAMPR3 scores. Studies have demonstrated that the inclusion of tryptophan within a peptide can increase its binding affinity and promote more extensive penetration into the cellular membrane, possibly due to the indole side-chain which has a stronger binding affinity with the interface area of lipid membranes, thus disrupting the nonpolar interactions of the lipid bilayer (Feng et al., 2020; Mishra et al., 2018). The efficacy of this phenomenon can be augmented by the adjacency of arginine cationic lateral chains in close proximity to the aromatic lateral chains of tryptophan, thereby intensifying cation-π interactions and yielding a peptide that is more energetically stable and securely embedded in the cellular membrane environment (Aliste et al., 2003, Gallivan and Dougherty, 1999). This pattern was observed in MM5, MM7, and indolicidin, which had the highest antimicrobial scores based on the CAMPR3 tool. Additionally, the incorporation of phenylalanine can promote favourable contacts between the aromatic ring of phenylalanine and the cell membrane, while also maintaining an extensive range of activity with low hemolytic activity (Lee et al., 2014). Indeed, there have been several studies in the literature examining the effect of adding positive charged residues to AMPs. For example, in a research conducted by Deslouches et al. (2015), the addition of positively charged residues to a peptide led to increased activity against *Pseudomonas aeruginosa* (Deslouches et al., 2015). Similarly, another study by Yeung et al. (2011) showed that adding positively charged residues to a peptide resulted in increased activity beside methicillin-resistant *S. aureus* (MRSA) (Yeung et al., 2011). In our study, we have also observed that the addition of positive charged residues to the VK6 peptide led to increased antimicrobial activity. Our results are consistent with previous studies and suggest that the addition of positive charged residues is a viable strategy to enhance the antimicrobial activity of peptides (Koo and Seo, 2019; Fjell et al., 2012; Mardirossian et al., 2014; Mishra et al., 2017). The addition of proline to the

designed peptides was found to have a relatively positive effect on increasing their antimicrobial activity compared to the original VK6 peptide. The rationale behind introducing proline was to create a kink in the peptide structure, which prevents the formation of a continuous alpha-helix. The disruption of the alpha-helical conformation may induce modifications in the peptide's mechanism of action, given its established participation in the interplay between AMPs and cellular membranes (Mishra et al., 2017). On the contrary, it has been explained that AMPs rich in proline exhibit activity that inhibits protein synthesis, as opposed to functions that disrupt the cell membrane (Mardirossian et al., 2018). For example, indolicidin has been revealed to hinder DNA synthesis and partially inhibit RNA and protein synthesis, while it can permeabilise bacterial membranes, lysis does not occur in bacterial cells (Subbalakshmi and Sitaram, 1998). It is worth acknowledging that the possession of an alpha-helical structure is not a universal trait among AMPs, as there exist alternative secondary structures that may confer antimicrobial activity.

The 3D structure prediction of the designed AMPs indicated a random coil folding pattern, which is similar to VK6 and indolicidin peptides. However, it has been reported that short peptides without disulfide bonds usually lack stable 3D structures, particularly those with a length of less than 35 amino acids (Subbalakshmi and Sitaram, 1998). Despite this, the initial physicochemical analysis showed acceptable stability of the designed AMPs, except for MM7 and indolicidin, which had instability indices greater than 40. To enhance the amphipathic nature of the designed AMPs, hydrophilic amino acids, such as threonine, were replaced with basic and amphipathic amino acids like arginine or lysine and tryptophan, respectively. Additionally, hydrophobic residues such as alanine and valine were substituted with phenylalanine and glycine, respectively, resulting in a significant change in the amphipathic structure of the designed AMPs, which was confirmed by the helical wheel diagram.

Given that *S. aureus*, *K. Pneumonia*, *P. aeruginosa*, and *E. coli* are common pathogens causing nosocomial and skin/soft-tissue infections (Khan et al., 2015) (Dryden, 2010; Bortolin et al., 2017), we assessed the microbicidal effect of the designed AMPs against other pathogenic agents, such as *S. cerevisiae* and *C. albicans*, using the DBAASP online server. The results showed that the addition of phenylalanine, isoleucine, and tryptophan improved the antimicrobial spectrum of MM7 compared to VK6. This improvement might be attributed to the higher number of tryptophan residues, which is a characteristic of Tryptophan-rich AMPs like indolicidin (Boparai and Sharma, 2020). However, the increase in positive net charge (+8) did not enhance the antimicrobial activity of MM1, MM2, MM3, MM4, and MM5. Therefore, we hypothesise that the combined effect of lysine and arginine with tryptophan residues, especially in MM7, led to an escalated antibacterial activity, as previously reported by Mollica et al. (2018). Additionally, the designed AMPs showed a significantly improved anti-tuberculosis activity compared to the original VK6. The primary mechanism underlying the antibacterial activity of AMPs is the perturbation of the cellular membrane via the creation of pores. Moreover, a multitude of anti-tubercular peptides exhibit activity against alternative intracellular targets, thereby conferring efficacy against *Mycobacterium tuberculosis*. As an illustration, the AMP indolicidin effectively eradicates *M. tuberculosis* through the perturbation of the cell membrane and its interaction with both DNA and RNA (Kościuczuk et al., 2012). It is believed that a significant contributing factor to the development of drug resistance lies in the inability of peptides (or pharmaceutical agents) to effectively traverse the cellular membrane.

Hence, in order to achieve efficacious intracellular eradication of *Mycobacterium*, which endures within host cells and exerts control over the host's immune reaction for its own benefit, it is imperative that anti-TB peptides possess the ability to infiltrate macrophages without harming to other cells (Sonawane et al., 2011). In this regard, the efficacy of anti-mycobacterial peptides has been established, owing to their strong attraction towards the cellular membrane, reduced immune response induction, and a wide spectrum of activities, encompassing engagements with alternative cellular sites such as enzymes, nucleic acids, and organelles (Khusro et al., 2016).

AMPs, aside from their bactericidal properties, display a diverse array of characteristics, including antifungal, anti-biofilm, antiviral, and anticancer activities. In this regard, the designed AMPs were found to possess all of these features. With respect to antifungal activity, all of the peptides, except MM7, exhibited relatively high antifungal activity when compared to VK6 and indolicidin. These peptides met the criteria for being considered AFPs, such as possessing a positive net charge, hydrophobicity, and amphipathicity (Fernández de Ullivarri et al., 2020). The potential mechanisms underlying AFP could conceivably encompass the impediment of 1,3-β-Glucan and Chitin biogenesis in the cellular wall, as well as inducing membrane permeability (Fernández de Ullivarri et al., 2020). The designed peptides also exhibited notable antiviral activity, as determined by the Meta-iAVP online tool, when compared to VK6 and indolicidin. The possible mechanisms for antiviral activity that target viral proteins include blocking viral entry and inhibiting specific viral enzymes (Agarwal and Gabrani, 2021). The efficacy of the designed AMPs were assessed through the utilisation of the dPABBs server, with a focus on their anti-biofilm properties, which revealed significant anti-biofilm activity in comparison to the original VK6 peptide. These features may be ascribed to their capacity to impede the development and adherence of biofilms, repress quorum sensing agents, and disrupt pre-existing biofilm structures (Di Somma et al., 2020). Furthermore, all of the designed peptides, except for MM1, demonstrated notable anticancer activity according to the results obtained from the AntiCP 2.0 server. In contrast, no anticancer activities were observed for VK6 and indolicidin, as inferred from the same server. It has been reported that peptides with anticancer properties are capable of inducing destabilisation of the membranes of cancerous cells. This is due to the fact that cancer cells tend to exhibit a higher degree of negative charge in comparison to their normal counterparts, leading to apoptosis and necrosis (Chiangjong et al., 2020; Sadraeian et al., 2013). Moreover, the designed AMPs (MM3, MM4, and MM6) showed antioxidant activity, which allows them to scavenge free-radical agents and potentially offer additional therapeutic benefits for cancer treatment. All of the designed peptides showed no allergenicity or antigenicity and had anti-inflammatory properties, which make them favorable as therapeutic agents.

In order to assess the cytotoxicity of AMPs against a spectrum of cellular entities, encompassing microbial cells such as *A. baumannii*, as well as cancer cells, it is imperative to scrutinise their capacity to permeate the cellular membrane. With respect to this matter, we conducted an in silico evaluation to appraise the cellular permeability potential of our peptides, and the results indicated that only MM2, MM3, and MM7 exhibited high-affinity uptake and could penetrate the cell membrane as suggested by AntiCP 2.0 and MLCPP servers. Our designed peptides, along with indolicidin, belong to Type-WR peptides with a wedge-shaped conformation (Rozek et al., 2000) and are situated near the membrane-water interface (Shagaghi et al., 2016). Indolicidin is capable of penetrating the bacterial cell membrane without inducing substantial lysis (Hsu et al.,

2005), potentially assisted by means of the high number of amino acid residues of arginine and tryptophan, which contribute to the properties of cell-penetrating peptides (Shagaghi et al., 2016). Furthermore, indolicidin can interact with the DNA duplex, selectively inhibiting DNA synthesis (Ghosh et al., 2014).

The present investigation highlights the derivation of innovative AMPs from the indigenous VK6 peptide discovered in the Komodo dragon's AMPs. Of all the peptides that were designed, MM1-7 displayed the most potent antibacterial activity. This can be attributed to the judicious modifications made to the amino acid sequence, which resulted in an increase in the overall positive charge and hydrophobicity. Furthermore, these modifications were made in such a way as to prevent any deleterious effects on normal eukaryotic cells, such as hemolysis or cytotoxicity.

5 Conclusions

In conclusion, AMPs represent a promising category of antimicrobial agents that offer potential in addressing the global challenge of antibiotic-resistant infections, particularly those caused by MDR *A. baumannii*. The design of AMPs inspired by Komodo dragons holds promise for developing novel and cost-effective AMPs with enhanced antimicrobial, anticancer, and anti-inflammatory activities, anti-biofilm formation, and reduced toxicity, antigenicity, and allergenicity, making them suitable for addressing infections caused by MDR *A. baumannii*. However, further in vitro and in vivo evaluations as well as clinical inquiries, are imperative for the definitive authentication of the formulated peptides.

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