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TURRITOXIN PAIAA SOURCE OF NOVEL ANTIMICROBIAL PEPTIDES AGAINST A. BAUMANNII

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ABSTRACT Antimicrobial Resistance (AMR) has been a silent pandemic. Newer antibiotics are required to treat drug-resistant pathogens. Antimicrobial peptides (AMPs) are promising next-generation antibiotics that are naturally produced in all living organisms. They are crucial components of the natural resistance framework of an organism. Small cationic peptides of 10-60 amino acid lengths have been successfully used as antibiotics. Marine ecosystems have an immense diversity of organisms and microorganisms that interact with each other, and thus have unique defense mechanisms. Marine cone snails are an untapped source of small, cationic AMPs. These snails secrete various proteinaceous venoms to deal with predators and preys. These cone snail venoms remain unexplored for their potential as antimicrobial peptides. The structural analysis of the venomous protein, Turritoxin PaIAa, from cone snails showed a high content of amino acids commonly found in AMPs. However, it did not exhibit any antibacterial activity. This protein was further investigated by overlap fractionation to identify hidden antimicrobial and cell-penetrating peptides. The unique peptide stretches obtained from PaIAa were evaluated against the top-priority ESKAPE pathogens. All peptides were active against *A. baumannii*. All peptides had cell-penetrating properties. These AMP candidates also showed promising physical, physicochemical, and pharmacokinetic properties. The AMP candidates identified in this study can be further evaluated by in vitro and in vivo testing.

KEYWORDS : Antimicrobial Resistance (AMR), Antimicrobial Peptides (AMPs), Acinetobacter baumannii, Pharmacokinetics

INTRODUCTION

Modern healthcare faces threats and challenges due to Antimicrobial Resistance (AMR) (Mestrovic et al., 2022). AMR occurs when morphological and biological changes in bacteria make antibiotics ineffective (Murray et al., 2022). According to estimates, this global pandemic is expected to cause 10 million deaths by 2050 (O'Neil, 2014). However, a recent study on death tolls due to AMR explained that the number crossed 4.5 million by 2019 (Aguilar et al., 2023). As the World Health Organization (WHO) has widely announced in its global report on surveillance, antimicrobial resistance has become one of the biggest threats to global public health in recent decades because of the extensive use of classical antibiotics in healthcare systems, animal production, and the community. If this problem is not well controlled, common infections or minor injuries can be life-threatening (Luong et al., 2020).

The World Health Organization (WHO) published the ESKAPE pathogen list, which needs immediate attention. This list includes *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. These organisms are common worldwide. Although a common pattern of AMR organisms has been identified worldwide, notable changes in the country-wise prevalence have been identified. Considering this, the WHO along with the Department of Biotechnology, India published Indian Priority Pathogen List (IPPL). In addition to ESKAPE pathogens, IPPL includes *Streptococcus pneumoniae, Enterococcus spp., Salmonella spp., Shigella spp., Coagulase-negative Staphylococcus spp., Hemophilus influenzae, Neisseria meningitis.* These organisms have also been grouped according to the priority of attention needed (Sharma & Anuj, 2019). According to the WHO recommendations, new antibiotics are needed to treat these organisms.

Acinetobacter is an opportunistic pathogen (Alrahmany et al., 2021). It is associated with ventilator-associated pneumonia, septicemia, and surgical wound infections, particularly in immunocompromised patients (Gallego, 2016). Estimates of the crude mortality rate for these infections in the United States range from 37% to 52% (Ma & McClean, 2021). The remarkable capacity to acquire resistance has led to the formation of multi-drug resistance isolates. Such isolates do not respond to the last line of treatment (Vrancianu et al., 2020). This underlines the need for alternative medicines against *A. baumannii* (Khalili et al., 2023).

Antimicrobial peptides (AMPs) are promising next-generation antibiotics (Elibe Mba & Innocent Nweze, 2022). AMPs are bioactive small peptides that are naturally produced by all living organisms, AMPs represent the first line of defense against fungi, viruses, and bacteria (Moretta et al., 2021). These peptide molecules are widespread entities that prevail in almost all types of life forms, varying from microorganisms and invertebrates (insects) to higher classes of organisms, such as higher plants and vertebrates (animals, amphibians). Thus, they are a crucial component of the natural resistance framework of an organism (Borah et al., 2021). Commonly, AMPs are–10-60 amino acids long and are cationic (Huan et al., 2020). Among the different sources of AMPs, the search for these molecules over the years has focused on terrestrial environments; however, the current trend is moving toward marine ecosystems because of their vast unexplored diversity, along with the potential discovery of new molecules and novel activities. Marine ecosystems have an immense diversity of organisms and microorganisms that interact with each other, and thus have to defend themselves against pathogens. Therefore, they present a wide range of mechanisms that enable them to survive in their natural environments (Bertrand & Munoz-Garay, 2019).

Venomous species from marine cone snails remain unexplored for their potential as antimicrobial peptides. Almost all marine snails within the superfamily Conoidea and Turridae produce proteinaceous venoms against their prey and predators (Marchot et al., 2024). Each species within these families can express approximately 50-200 peptide toxins, consisting of multiple disulfide cross-links and distinct post-translational modifications (Olivera et al., 2014). Many toxins secreted by the Conidae family have been well characterized. However, toxins from the family Turridae have been studied considerably less than their Conidae counterparts because of their small size and tedious toxin extraction procedures (Turner et al., 2018). Therefore, turrid toxins (turritoxins) have been barely described (Hernández-Sámano et al., 2020).

Historically, the composition of toxins from snails has shown the presence of over hundred peptides. In contrast, transcriptomic analysis of *P. albida* (Polystira genus) showed the presence of only three peptides, namely PaIAa, pal9a, and Pal9.2 (Hernández-Sámano et al., 2020). Limited information is available for the latter two peptides.

PaIAa is an 85 aa long protein isolated from White Giant Turris or *Polystira albida,* which is toxic to *Drosophila* larvae (López-Vera et al., 2004). The 85 a.a. full length sequence does not show antibacterial

activities. However, sequential insights of PaIA suggest an abundance of commonly found amino acid residues in AMPs such as Alanine, Cystine, Glycine, Histidine, Isoleucine, Lysine, Leucine, Proline, Arginine, and Valine (Decker et al., 2022). This suggested that the PaIAa sequence may contain novel AMPs.

This hypothesis about the less explored protein, Turritoxin PaIAa, was investigated further by overlap fractionations to identify hidden antimicrobial and cell-penetrating peptides. The unique peptide stretches obtained from PaIAa were evaluated against the top-priority ESKAPE pathogens. All peptides were active against *A. baumannii*. All peptides had cell-penetrating properties. These promising AMP candidates were also tested for their physical, physicochemical, and pharmacokinetic properties and were determined to suggest candidates with optimum antibacterial activities.

MATERIALS AND METHODS:

Screening for suspected Antimicrobial Peptides from Turritoxin isolated from *Polystira albida*

CAMPR4 was used to screen for AMPs of 10-20 a.a. lengths from the 85 amino acid original sequence (Accession number: P0C1X4). Natural amino acids were selected to generate the AMPs. These generated AMPs were evaluated for antimicrobial properties for the activity against bacterial genomes using DBASSP (accessed on 26.02.2024).

Estimating Physical Characteristics of the Peptides:

Physical Characteristics are primary indicators of drug suitability. Therefore, molecular Weight, Theoretical pI, and total Number of Positive and Negative Charges were estimated using Expasy Protparam (as accessed on 02-03-2024). Because peptides are active against bacterial genomes, it is essential to determine their cell-penetrating properties. This was done with the help of CellPPD (accessed on 12.03.2024). Furthermore, the fitness of these candidates was checked using physical, chemical, and ADMET properties.

Evaluation of Physicochemical Characteristics of the peptides

In addition to antibacterial and physical activities, physicochemical characteristics help to identify the stability and potential activities against Human RBCs and undesirable immune responses. The Hemolytic Activity of the peptides was checked using HemoPI (accessed on 26.02.2024). Other characteristics, such as stability under standard conditions, hydrophobic/hydrophilic nature, and thermostability of the peptides, were checked using Expasy Protparam (accessed on 27.02.2024), and The Boman Index was checked using the APD3 tool. The peptides' ability to induce Class I immunogenicity was assessed using IEDB (as accessed on 29-02-24).

Elucidating the Pharmacokinetics properties of the selected peptides

Drug administration, dissolution, and distribution within the human body are some important parameters that determine the success of a potential molecule as a drug. Additionally, the potential toxicity to different human cells and carcinogenicity are vital for detailed AMP studies. The selected peptides' ADMET properties (Adsorption, Distribution, Metabolism, Excretion, and Toxicity) were elucidated using ADMETlab2.0 (accessed on 08.03.24). ADMET lab 2.0 requires inputs in the form of SMILE structures. SMILES were generated by providing AMP sequence inputs in pepSMI (accessed on 08.03.24)

Peptides	Escherichia coli ATCC 25922	Pseudomonas aeruginosa ATCC 27853	Klebsiella pneumoniae ATCC 700603	Salmonella typhimurium ATCC 14028	Acinetobacter baumannii ATCC 19606	Staphylococcus aureus ATCC 25923	Enterococcus faecalis ATCC 29212
TURR_10_1	✓	X	X	✓	✓	X	X
TURR_13_1	X	X	X	X	✓	X	X
TURR_13_2	X	X	X	X	✓	X	X
TURR_15_1	X	X	X	X	✓	X	X
TURR_15_2	✓	X	X	X	✓	X	X
TURR_15_3	✓	X	X	X	✓	X	X
TURR_17_1	✓	✓	✓	✓	✓	X	X

Table 1: Antimicrobial Activity of the peptides for action on bacterial genome Key: Turr: Turritoxin (proposed name for the toxic proteins isolated from Turrids), length of AMP, and Sequence number

RESULTS:

Screening for suspected Antimicrobial Peptides from Turritoxin isolated from *Polystira albida*:

The CAMPR4 tool generated overlapping potential AMPs from 85 a.a. Turritoxin sequence. Candidates with a length range of 10-20 a.a. and AMP prediction scores above 0.7 were considered for this study (Table 1). It was also observed that the peptides with the highest scores were found in the C-terminus as compared to the N-terminus of Turritoxin PaIAa. The generated AMPs were active against bacterial genomes. All AMP motifs showed activity against *Acinetobacter baumannii* ATCC 19606 and were inactive against *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. Some peptides showed broad-spectrum activity. Peptide 'TURR_17_1' showed activity against all tested organisms, except *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. The antimicrobial activities of these peptides against bacterial genomes are summarized in Table 1.

Estimating Physical Properties of the Peptides

Physical properties, such as molecular weight, theoretical pI, and net charge, play a crucial role in determining antibacterial properties. Electrostatic interactions between AMPs and bacterial envelopes determine their antibacterial activity. As bacterial envelopes are negatively charged, high cationic charges on AMPs help them adhere better to bacterial membranes. Positive charge and pI above 6.0 are indicators of a good AMP candidate. As summarized in Table 2, all candidates were found to have charges above +4 and pI above 10. Because the peptides act on bacterial genomes, their cell-penetrating properties were evaluated using CellPPD. Each candidate exhibited the possibility of cell penetration. Thus, all the selected candidates are a good fit for AMPs.

Evaluation of Physicochemical Characteristics of the Peptides

The in vivo effects on human RBCs should be elucidated for any peptide to be further evaluated for pharmacokinetics. Potential drug candidates should have minimal or no activity on human erythrocytes. All AMPs tested showed intermediate hemolysis, with PROB scores below 0.5 (Fig 1a).

Ideally, a peptide should be stable under standard conditions, and the stability of the selected peptides under standard conditions was calculated as the instability index (Fig 1b). Two AMPs, TURR_10_1 and TURR_15_3, had instability indices below 40. This indicated that the peptides were stable. Other candidates have stability indices nearing 40-50 and thus are less stable than TURR_10_1 and TURR_15_3 under standard conditions. The aliphatic index is directly proportional to the thermostability of the peptides, that is, the higher the index, the higher is the thermostability (Li et al., 2016). TURR_13_1 had the lowest aliphatic index, indicating very low thermostability, while TURR_13_2, TURR_15_1, and TURR_15_2 showed moderate scores indicating moderate thermostability. The rest showed high scores and were thermostable.

The Hydrophobic and Hydrophilic nature of the peptides was calculated as the Grand Average Of Hydrophobicity (GRAVY) scores (Fig 1d). This is the ratio of the average hydrophobicity of a peptide to its length. Negative and positive values indicate globular (hydrophilic) and membranous protein (hydrophobic) peptides, respectively (Chang & Yang, 2013; Kyte & Doolittle, 1982). GRAVY scores for all peptides were negative, indicating their highly hydrophilic nature.

The Boman Index is an indicator of the interaction between peptides and other proteins. Most of the peptides had a Boman Index in the ranges of 0-3 kcal/mol. An index value above 3 kcal/mol indicates peptide multifunctionality (Boman H., 2003). A score of 4.56 kcal/mol suggests the multifunctionality of peptide TURR_10_1. The other

peptides had values below or near 3 kcal/mol. Therefore, the other candidates were not multifunctional.

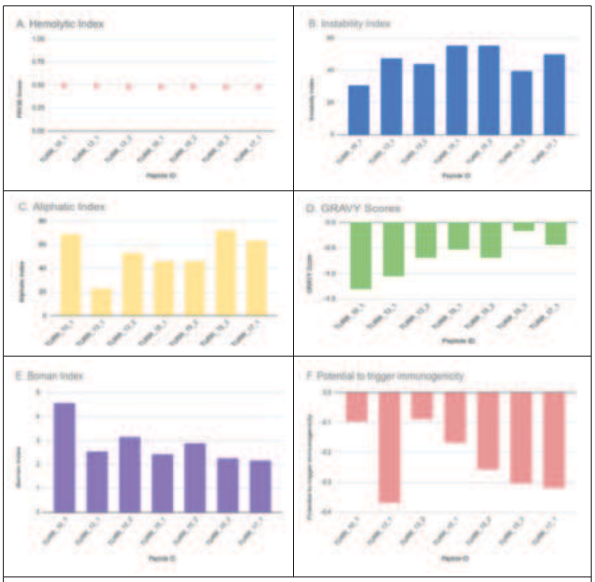

Fig 1: Physicochemical properties of selected AMPs: a) Hemolytic Index of the peptides. The PROB score indicates the hemolytic activities of the peptides (0-1). '0' indicates least hemolytic activity and '1' being the most hemolytic. b) Indicates instability indexes of the peptides at standard conditions. Candidates with scores >40 are considered unstable. c) The heat stability of the peptides is calculated using an aliphatic index. The aliphatic indexes are directly proportional to heat stability d) Hydrophobic and Hydrophilic nature of peptides Negative and positive values indicate globular (hydrophilic) and membranous proteins (hydrophobic) peptides respectively. e) The interaction of peptides with other proteins was calculated as Boman Index. Values higher than 0.3 indicate the multifunctional nature of peptides. f) The probability of the peptides triggering Class I immunogenicity was estimated.

Peptide molecules can trigger immune responses. The laborious, time-consuming, and costly process of wet lab analysis can be avoided by in silico predictions. The potential of the peptides to elicit a Class I Immune Response was assessed using IEDB. All peptides showed negative index values. Values below 0.3 indicate a non-immunogenic nature (Kuriakose et al., 2016; Shankar et al., 2014). Hence, all AMP candidates in this study were non-immunogenic.

Elucidating the Pharmacokinetics Properties of the Selected Peptides

The drug development process can be facilitated by computational evaluation of the pharmacokinetic properties of a potential drug. These properties include Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET), carcinogenicity, and skin sensitivity/irritation (Okella et al., 2022). The selected peptides were tested for all such properties using ADMET lab 2.0 (Xiong et al., 2021).

The degree of drug absorption by intestinal cells was assessed using human intestinal cells (HIA) and Human Colon Adenocarcinoma Cell lines (CaCo2). The optimal values for the absorption in HIA and CaCo2 are above 0.3 and -0.5, respectively. All peptides can be easily absorbed by HIA and CaCo2 cells (Table 3) (Basant et al., 2016).

One of the main mechanisms of drug uptake is its distribution throughout the body. This is tested on three parameters such as volume distribution (VD), ability to cross Blood Brain Barrier (BBB), and Plasma Protein Binding (PPB) capacities. Optimum values for VDss are between 0.04 - 20 L/Kg, for BBB positive ≥0.1≤ negative, and for PPB less than 90% indicates a high therapeutic index whereas greater than 90% low therapeutic index. VDss was within the permissible range for all peptides.

The Volume distribution describes the in vivo distribution of drugs with an optimal range of 0.04-20 L/Kg. All chosen peptides have a volume distribution within the desired range. Drugs that act in the CNS must cross the blood-brain barrier (BBB) to reach their molecular targets. In contrast, for drugs with a peripheral target, little or no BBB penetration may be required to avoid CNS side effects. The optimal range for BBB Values was 0-0.3 cm/s. All peptides had desirable BBB values, indicating a lower chance of triggering CNS Side Effects. The binding of any potential drug to plasma has a strong influence on its pharmacodynamic behavior and should be checked. PPB or Plasma Protein Binding values of the peptides were checked, which showed values in the range of 14-28% indicating that the peptides have a high therapeutic index.

Peptide ID	Absorption		Distribution			Metabolism				Excretion		Toxicity	
	HIA	CaCO2	PPB	BBB	VD	CYP1A2 inhibitor	CYP1A2 substrate	CYP3A4 inhibitor	CYP3A4 substrate	CL	T1/2	Skin sensitivity	Carcinogenicity
TURR_10_1	0.857	-6.482	14.46%	0.05	0.513	0	0	0	0	1.026	0.628	0.105	0.034
TURR_13_1	0.95	-6.408	21.56%	0.033	0.522	0	0	0.001	0	0.902	0.819	0.106	0.038
TURR_13_2	0.966	-6.988	17.83%	0.024	0.408	0	0	0	0	0.62	0.74	0.123	0.019
TURR_15_1	0.957	-6.422	20.83%	0.017	0.431	0	0	0	0	0.706	0.776	0.09	0.013
TURR_15_2	0.963	-6.323	24.87%	0.021	0.435	0	0	0	0	0.655	0.814	0.089	0.029
TURR_15_3	0.985	-6.809	19.41%	0.01	0.382	0	0	0	0	0.542	0.772	0.078	0.025
TURR_17_1	0.996	-6.467	27.77%	0.009	0.364	0	0	0	0	0.502	0.828	0.075	0.02

Table 3: Evaluation of Absorption, Distribution, Metabolism, Excretion, and Toxicity of the chosen peptides: HIA: Human Intestinal Absorption: HIA > 0.3: HIA positive HIA < 0.3: HIA negative, CaCO2: Optimal higher than -5.15 Log Unit
PPB: Plasma Protein Binding (<90% Drugs with High Protein Bound may have Low Therapeutic Index) BBB: Blood Brain Barrier Penetration: 1 being BBB+ and 0 being BBB-
VD: Volume Distribution: Optimal: 0.04-20L/Kg,
Metabolic Properties of the chosen peptides: >0.5: inhibitor <0.5: non inhibitor and >0.5: substrate <0.5: non-substrate
CL: Clearance: High: >15 Moderate: 5–15 Low: <5, T1/2: Half Life: Long half-life: >3 h Short half-life: <3 h

CL represents the drug clearance. All peptides showed low clearance rates. The half-life of the peptides was observed in the ranges 0.6-0.83 indicating a longer half-life for each peptide. Skin sensitization is a potential adverse effect of dermally applied products. None of the peptides showed any risk of triggering an allergic response in the skin. Carcinogenicity is of great concern owing to its serious effects on human health. None of the peptides showed carcinogenic activity. Drug efficacy is based on the ability to act as a substrate or inhibitor of the human cytochrome P450 family. All peptides were found to be non-inhibitors.

DISCUSSION:

Turritoxin derived from Gastropod Turrid *Polystira albida*, was found to be a potential protein containing AMP stretches. All the selected AMP motifs showed activity against *Acinetobacter baumannii* ATCC 19606. Peptide 'TURR_17_1' showed activity against all test organisms except *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 in the bacterial genome approach. 'TURR_17_1' has satisfactory ADMET Results and a broad spectrum of activity which could make it a potential drug candidate in the future. The activities of this drug can be explored using *in vitro* and *in vivo* assays. A targeted approach for peptide drug development from toxic proteins using machine learning was employed. These predicted toxin-derived peptides can also be used for cargo delivery and drug development (De Cena et al., 2022).

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