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ORIGINAL PAPER

In-silico homology modeling of three isoforms of insect defensins from the dengue vector mosquito, *Aedes aegypti* (Linn., 1762)

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Abstract Dengue is a serious public health problem in tropical and subtropical countries. It is caused by any of the four serologically distinct dengue viruses, namely DENV1–4. The viruses are transmitted by Aedes mosquitoes. Understanding various defence mechanisms of insects has become a prime area of research worldwide. In insects, the first line of defence against invading pathogens includes cellular mechanisms and a battery of antimicrobial peptides such as defensins, cecropins etc. Defensins—cationic, cysteine-rich peptides consisting of ~40 amino acids with broad-spectrum activity against Gram-positive bacteria—have been reported from a wide range of organisms. In the dengue vector mosquito, *Aedes aegypti*, three isoforms of defensins are reported to be expressed in a spatial and temporal fashion. This report presents the three-dimensional structures of the three isoforms of *Ae. aegypti* defensins predicted by comparative modeling. Prediction was done with Modeller 9v1 and the structures validated through a series of tests. The best results of the prediction study are presented, and may help lead to the discovery of new synthetic peptides or derivatives of defensins that could be useful in the control of vector-borne diseases.

Keywords *Aedes aegypti* · Defensins · Homology modeling · Modeller · Procheck

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Introduction

Dengue is one of the most serious public health problems in tropical and subtropical countries, especially in Southeast Asia [1]. Dengue is caused by one of four serologically distinct dengue virus (DENV) namely DENV1–4 [2]. These viruses are transmitted from human to human primarily by the mosquitoes *Aedes aegypti* and *Ae. albopictus* [3]. Ecological and climatic factors are known to influence the prevalence and spread of the vector mosquitoes [4, 5]. Increase in global temperature could spread the virus to new areas previously devoid of it, as proliferation of virus increases with temperature rise [6].

Antimicrobial peptides are important components of the innate immunity of a wide range of organisms and present the first line of defence against invading microorganisms. Typically cationic, the peptides act against bacteria, fungi, and enveloped viruses through mechanisms involving membrane disruption or pore formation leading to leakage of cell content and destruction [7, 8].

Defensins are short cationic immune peptides consisting of ~40 amino acids, characterised by the presence of 6–8 cysteine residues [9]. They have been recorded from a wide range of organisms from amoeboid protozoan [10] to humans and are being extensively studied in insects, especially in disease-

causing vector mosquitoes. In 1989, Lambert et al. [11] first reported defensins from the dipteran insect *Phormia terranovae* and proposed the term 'Insect Defensin'. Insect defensins act against a wide range of Gram-positive bacteria whereas against few Gram-negative bacteria. In the mosquito body these peptides have been reported to be expressed at sites of primary infection such as mid-gut epithelium, respiratory tracheal system etc., and also in fat bodies and haemocytes. Defensins are reported to exist in isoforms based on their spatial and temporal expression pattern.

In *Ae. aegypti*, the dengue/chikungunya vector mosquito, three isoforms of insect defensins viz., Defensin A, Defensin B [12] and Defensin C [13], have been reported (Fig. 1) and their sequences are available in the NIH sequence database (www.ncbi.nlm.nih.gov/). In immune-activated *Ae. aegypti*, isoforms A and B were found in equal amounts whereas isoform C was found at levels only one-fifth of that of isoforms A and B. In addition, the signal peptide and pro-defensin regions of isoforms A and B are similar, thus A and B are believed to be allelic variants of one gene. Isoform C, however, has a different length signal peptide, and modifications within the signal peptide and propeptide region. These genes also have a distinct transcription profile: only transcripts for defensin C were found in the midguts of naive *Ae. aegypti* whereas isoforms A/B were transcribed very strongly in the fat body after immune activation. These differences in haemolymph concentration and transcription site may reflect tissue-specific expression patterns of the different genes [9].

Structure prediction by homology modeling (HM) can aid understanding the three dimensional (3D) structure of a given protein. This in turn will help elucidate the mechanisms behind protein function, since function is determined by 3D structure [14, 15]. HM has proved very useful in the prediction of the 3D structure and function of insect proteins, especially those important in public health, e.g. proteins from filarial vector mosquitoes such as *Culex quinquefasciatus* [16] and *Ae. aegypti* [17], as well as those from viruses such as dengue virus [18, 19] and West Nile virus [20–23]. Modeling of an amino acid sequence based on known structures consists of four steps: finding known structures related to the sequence to be modeled (i.e. templates), aligning the sequence with the templates, building a model, and assessing the model. Comparative structure prediction produces an all-atom model of a sequence based on its alignment to one or more related protein structures. Comparative model building includes either sequential or simultaneous modeling of the core of the protein, loops, and side chains [24]. Most antimicrobial peptides, including defensins, act against microbes by forming pores or ion channels in the membrane of the target organisms. This is accomplished by the structure of these molecules, which form oligomers [25–27]. In the present study, the 3D structures of the three defensin isoforms from the vector mosquito *Ae. aegypti* have been predicted by HM. These predicted 3D structures might help understand the function of insect defensin isoforms in vector mosquitoes.

Materials and methods

Search and retrieval of sequences and structure

The nucleotide sequences of the three isoforms of defensins viz., Defensin A, B and C were retrieved from

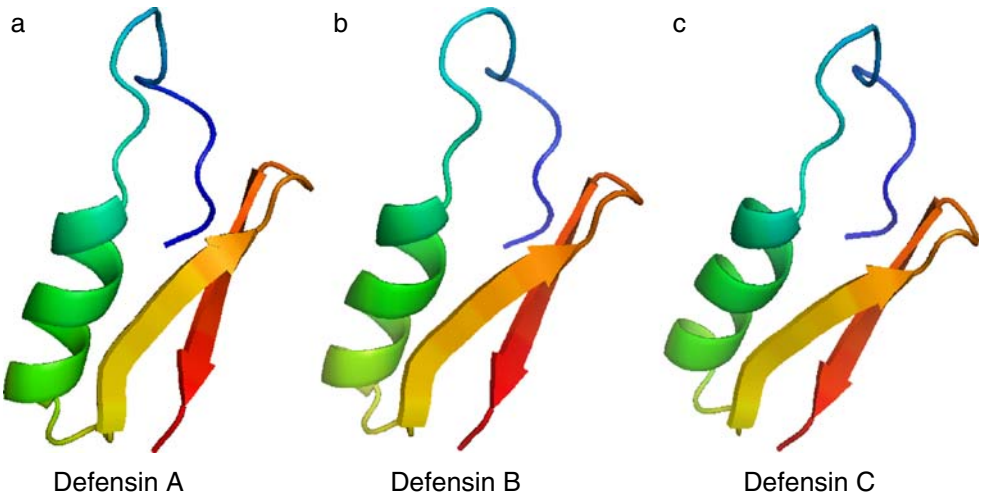


Fig. 1 Predicted structures of the isoforms of defensins from *Aedes aegypti*

the NIH webpage (www.ncbi.nlm.nih.gov/). The accession numbers are as follows: 2121486A, 2121486B and AAB35031. The basic local alignment search tool for protein (BLASTp) [28] search (www.ncbi.nlm.nih.gov/blastp) was performed for these sequences with the protein data bank (PDB) to identify homologous proteins with available structure.

Selection of template

Multiple sequence alignment analysis of the three defensin peptides from *Ae. aegypti* and the two from flesh fly was performed with ClustalW [29, 30] (http://www.ebi.ac.uk/).

To investigate how closely related the sequences of the two available structures are, a phylogenetic analysis was done with the 'Proml' module of the PHYLIP 3.66 (http://evolution.gs.washington.edu/phylip.html) package [31]. 'Proml' estimates phylogenies from protein amino acid sequences by maximum likelihood. The Jones, Taylor and Thornton (JTT) model [32] was employed. Constant rate of change was used, without weighted sites, inferring which sites have which rates.

Homology modeling

Homology modeling was done using Modeler 9v1 [33, 34]. The co-ordinate file from PDB was used as such; alignment files were prepared from the ClustalW output and scripts were written to predict five models.

Evaluation of predicted models

All 15 predicted models (5 per sequence) were evaluated by Procheck [35] (http://www.biotech.ebi.ac.uk/) performing full geometric analysis with a resolution of 1.5 Å. Ramachandran plot statistics was used to evaluate the best model.

The root mean square deviation (RMSd) values were calculated using the Swiss pdb viewer (http://www.expasy.ch/spdbv/) by fitting the carbon backbone of the predicted model onto the template structure.

The total, local, burial and contact energies of the predicted models were calculated using predicted structure quality score (PSQS) [36–39] (http://www1.jcsg.org/cgi-bin/psqs-cgi/cos). PSQS is an energy-like measure of the quality of protein structure. It is calculated based on the statistical potentials of mean force describing interactions between residue pairs and between single residues and solvent. Three sets of statistical potentials were derived from the statistics calculated for a representative set of 1,836 proteins containing all domain folds available in the SCOP database. Local potential describes the local geometry propensities of the polypeptide chain. It is a function of residue types of residues i − 1, i and i + 1. Burial potential describes the tendency of hydrophobic residues to concentrate in the protein core, and the tendency of hydrophilic residues to concentrate on the protein surface. Contact potential describes the likelihood of different residue pairs to be in close contact. PSQS makes it possible to differentiate between correct and incorrect protein structures. It may also be useful during structure refinement to locate the places that are significantly different from typical geometries. The average PSQS for structures covering all folds taken from the SCOP database is −0.27 and most structures have a PSQS value of less than −0.1.

Taking into account all these parameters, one best model was selected from the five predicted models for each peptide.

Results and discussion

BLASTp search revealed two crystallographic structures; 1ICA—the NMR structure of insect defensin A from the flesh fly *Phormia terranovae* [40], and 1L4V—the NMR structure of the sapecin molecule from the flesh fly (*Sarcophaga peregrina*) [41]. 1ICA and 1L4V had 10 and 18 NMR structures, respectively.

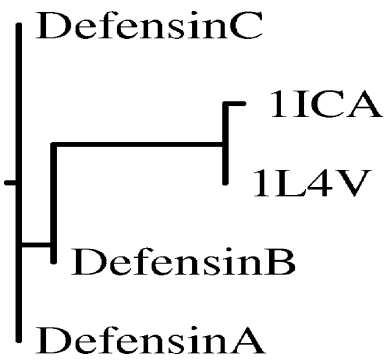
Multiple sequence alignment analysis by ClustalW revealed that the amino acid residues at positions 9, 11, 12, 13, 21, 22, 24, 32 and 34 of the target sequences varied from both the template sequences; 1L4V has an alanine residue at position 34 whereas 1ICA has a glycine residue at that position.

```
DefensinA       ATCDLLSGFG VGDSACAAHC IARRNRGGYC NAKKVCVCRN  Target
DefensinC       ATCDLLSGFG VGDSACAAHC IARRNRGGYC NAKKVCVCRN  Target
DefensinB       ATCDLLSGFG VGDSACAAHC IARGNRGGYC NSKKVCVCRN  Target
1L4V            ATCDLLSGTG INHSACAAHC LLRGNRGGYC NGKAVCVCRN  Template
1ICA            ATCDLLSGTG INHSACAAHC LLRGNRGGYC NGKGVCVCRN  Template
```

Phylogenetic analysis of the five defensin molecules based on the 'Amino acid sequence Maximum Likelihood method, version 3.66' of PHYLIP v. 3.66 showed that 1L4V was more closely related to defensins from *Ae. aegypti* than 1ICA.

Jones-Taylor-Thornton model of amino acid change

```
+Defensin C
 |                +1ICA
 |                |
 |  +-------------3
 1--2             +1L4V
 |   +Defensin B
 |
 |
+Defensin A
```



Remember: this is an unrooted tree!

Ln Likelihood = -179.58140

Between	And	Length
1	Defensin A	0.00010
1	Defensin C	0.00010
1	2	0.05274
2	3	0.25893
3	**1ICA**	**0.02644**
3	**1L4V**	**0.00010**
2	Defensin B	0.00010

Based on the results of multiple sequence analysis and phylogenetic analysis, structure 1L4V was selected as the template for modeling the target sequences. The 12th conformer was claimed to be the best representative of the ensemble by the authors and therefore, this conformer was used as the template.

Homology model building was done using Modeller v 9.1. The modeled structures of isoforms of defensins from *Ae. aegypti* had one helix and a pair of anti-parallel β-sheets interlinked by coils. The helix was present on the N-terminal region preceded by a relatively long coil; β-sheets occupied the C-terminal region and were interlinked among themselves and to the helix by folds. The structures were visualized using Pymol [42].

The predicted structures were validated with Procheck, performing full geometric analysis, which involved calculation and analysis of main chain bond lengths and bond angles, plotting of bond angles (Ramachandran plot), calculation and analysis of the stereochemistry of main and side chains and calculation of G factors, thereby assessing the quality of the prediction. The Ramachandran statistics, RMSd and PSQS of the predicted structures for each isoform are consolidated in Tables 1, 2 and 3. The PSQS for the template was calculated to be: Local −0.1528; Burial 0.0367; Contact −0.1140 and Total −0.2300.

Defensin A

The amino acid sequence of the defensin A peptide from *Ae. aegypti* has five glycine residues. Therefore, 33 residues were taken into account for plotting the Ramachandran plot, leaving the two terminal residues (Fig. 2).

Table 1 Defensin A (total of 33 residues). Most favoured structure shown in *bold*. *RMSd* Root mean square deviation

Predicted structure	Ramachandhran statistics					RMSd value	Calculated energies of predicted structures			
	No. of residues in:									
	Most favoured region (M)	Additional allowed region (A)	Generously allowed region (G)	Disallowed region (D)	Percentage (M/n)		Local	Burial	Contact	Total
1	29	3	1	0	87.9 (29/33)	0.48 Å	−0.1435	0.063	−0.1865	−0.267
2	28	3	1	1	84.8 (28/33)	0.38 Å	−0.1435	0.0582	−0.1817	−0.267
3	29	2	2	0	87.9 (29/33)	0.48 Å	−0.1435	0.0752	−0.197	−0.2652
4	**29**	**3**	**1**	**0**	**87.9 (29/33)**	**0.44 Å**	**−0.1435**	**0.0625**	**−0.1953**	**−0.2763**
5	27	4	1	1	81.8 (27/33)	0.52 Å	−0.1237	0.063	−0.1935	−0.2542

Table 2 Defensin B (total of 32 residues). Most favoured structure shown in *bold*

Predicted structure	Ramachandhran statistics					RMSd value	Calculated energies of predicted structures			
	No. of residues in:									
	Most favoured region (M)	Additional allowed region (A)	Generously allowed region (G)	Disallowed region (D)	Percentage (M/n)		Local	Burial	Contact	Total
1	**29**	**2**	**1**	**0**	**90.6 (29/32)**	**0.50 Å**	**−0.1723**	**0.0505**	**−0.179**	**−0.3007**
2	28	3	1	0	87.5 (28/32)	0.48 Å	−0.1723	0.0602	−0.1865	−0.2985
3	28	3	1	0	87.5 (28/32)	0.46 Å	−0.1723	0.051	−0.1955	−0.3167
4	28	3	1	0	87.5 (28/32)	0.42 Å	−0.1723	0.0505	−0.186	−0.3077
5	27	2	3	0	84.4 (27/32)	0.40 Å	−0.1957	0.0365	−0.179	−0.3382

Table 3 Defensin C (total of 33 residues). Most favoured structure shown in *bold*

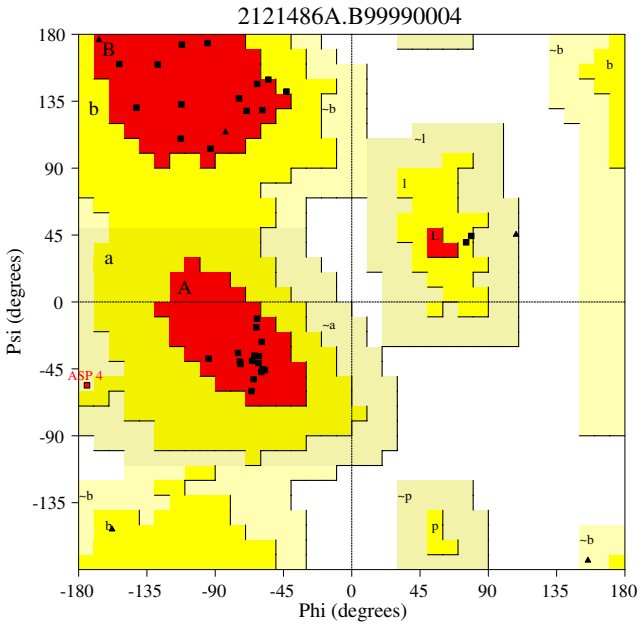
Predicted structure	Ramachandhran statistics					RMSd value	Calculated energies of predicted structures			
	No. of residues in:									
	Most favoured region (M)	Additional allowed region (A)	Generously allowed region (G)	Disallowed region (D)	Percentage (M/n)		Local	Burial	Contact	Total
1	29	3	1	0	87.9 (29/33)	0.48 Å	−0.1435	0.063	−0.1865	−0.267
2	28	3	1	1	84.8 (28/33)	0.38 Å	−0.1435	0.0582	−0.1817	−0.267
3	29	2	2	0	87.9 (29/33)	0.48 Å	−0.1435	0.0752	−0.197	−0.2652
4	**29**	**3**	**1**	**0**	**87.9 (29/33)**	**0.44 Å**	**−0.1435**	**0.0625**	**−0.1953**	**−0.2763**
5	27	4	1	1	81.8 (27/33)	0.52 Å	−0.1237	0.063	−0.1935	−0.2542

Table 1 shows the consolidated validation results of the five predicted structures of defensin A. Considering the maximum number of residues falling in the most favoured region, least RMSd and energy values, the fourth predicted structure (values in bold in Table 1) was considered to be the best model for defensin A of *Ae. aegypti* (see Fig. 1a).

The helix of the predicted structure for defensin A had approximately three turns comprising ten residues spanning from Ser14 to Arg23, all falling in the core alpha region. Both the sheets comprised five residues each, spanning from Gly27 to Asn31 and Val35 to Arg39, respectively. Residues Tyr29 and Asn31 of the first sheet lie in the core beta region, while Cys30 lies in the additional allowed beta region. The other two residues were Gly27 and Gly28. On the other hand, with the exception of Cys36, all four residues of second sheet lie in the core beta region.

Defensin B

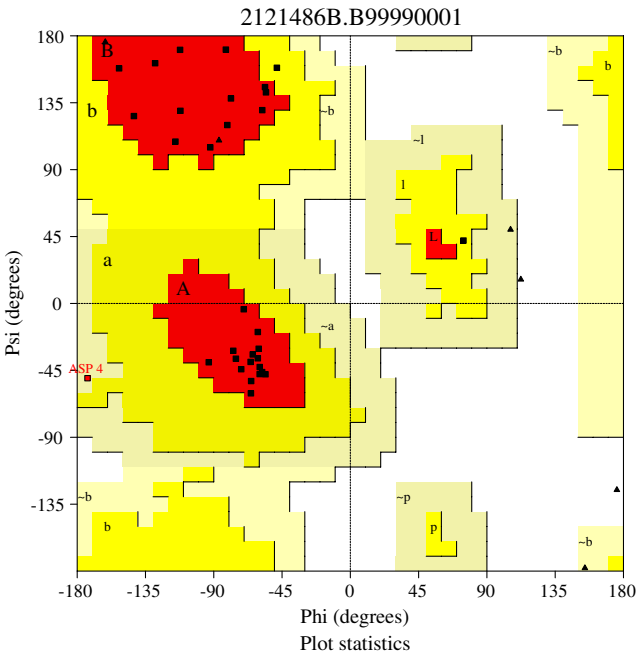
The amino acid sequence of the defensin B peptide from *Ae. aaegypti* has six glycine residues. Therefore, 32 residues were taken into account for plotting the Ramachandran plot, leaving the two terminal residues (Fig. 3).



Plot statistics

Residues in most favoured regions [A,B,L]	29	87.9%
Residues in additional allowed regions [a,b,l,p]	3	9.1%
Residues in generously allowed regions [~a,~b,~l,~p]	1	3.0%
Residues in disallowed regions [XX]	0	0.0%
Number of non-glycine and non-proline residues	33	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	5	
Number of proline residues	0	
Total number of residues	40	

Fig. 2 Ramachandran plot of the predicted structure of defensin A (structure 4) from *Aedes aegypti*



Plot statistics

Residues in most favoured regions [A,B,L]	29	90.6%
Residues in additional allowed regions [a,b,l,p]	2	6.2%
Residues in generously allowed regions [~a,~b,~l,~p]	1	3.1%
Residues in disallowed regions [XX]	0	0.0%
Number of non-glycine and non-proline residues	32	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	6	
Number of proline residues	0	
Total number of residues	40	

Fig. 3 Ramachandran plot of the predicted structure of defensin B (structure 1) from *Ae. aegypti*

The results of validation of predicted structures of defensin B are consolidated in Table 2. Among the five predicted structures, the first prediction (see Fig. 1b) had the maximum number of residues (29 residues) falling in the most favoured region when compared to 28 and 27 residues for the other four predicted structures. Though the RMSd value of prediction 1 was 0.1Å higher than the least RMSd (predicted structure 5), it was well within the range [43]. Also, the calculated total energy of predicted structure 1 was lower than the average PSQS of −0.27 of the 1,836 proteins from the SCOP database.

The secondary structures of defensin B were identical to those of defensin A since both the peptides had identical sequences in those regions.

Defensin C

The primary structure of defensin C was identical to that of defensin A. Therefore, the structure prediction and qualities were also identical as can be seen from Table 3 and Fig. 4.

Since the validation statistics of defensin C were identical to those of defensin A, predicted structure 4, which proved to be the best model, was selected (see Fig. 1c).

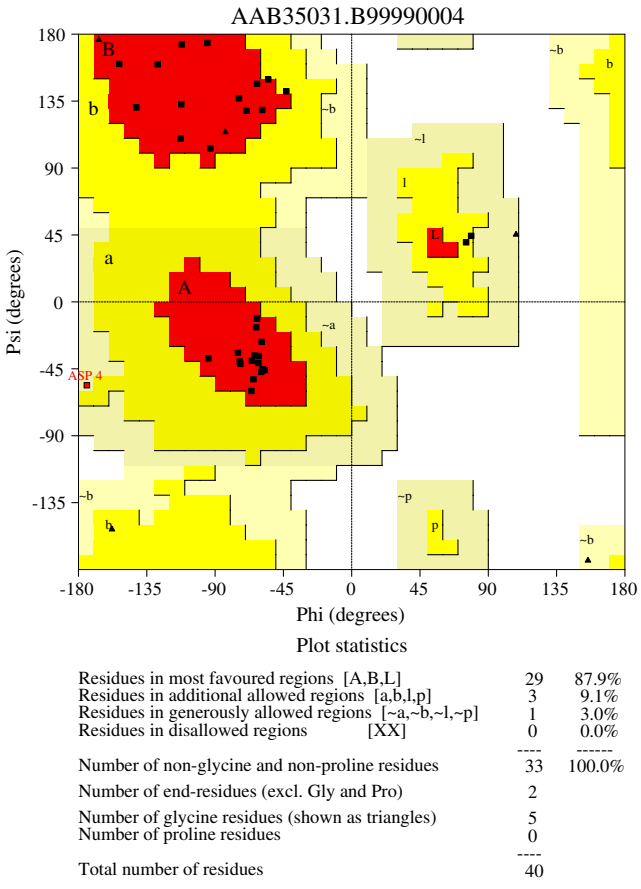


Fig. 4 Ramachandran plot of predicted structure of defensin C (structure 4) from *Ae. aegypti*

Concluding remarks

Many derivatives of antimicrobial peptides (AMPs) are now being developed and tested for activity against micro-organisms. Colimycin, the methosulfate derivative of the cationic lipopeptide colistin (polymyxin E), has been utilised quite successfully in an aerosol formulation against *Pseudomonas aeruginosa* lung infections. rBPI21 is a synthetic AMP produced by Neuprex (Xoma, Berkeley, CA). In a phase II/III clinical trial of therapy against meningococcemia, rBPI21 given intravenously along with other supportive therapies resulted in a dramatic decrease in deaths. Another well-studied peptide is the magainin derivative MSI-78 produced by Locilex (Magainin Pharmaceuticals, Plymouth Meeting, PA). In a clinical trial of 926 patients, topical MSI-78 was found to show equivalence to oral ofloxacin against polymicrobic diabetic foot ulcers. Nisin (a lantibiotic cationic peptide produced by AMBI, Purchase, NY) and IB-367 (a protegrin-like cationic peptide from Intrabiotics, Mountain View, CA) have undergone phase I (safety) clinical trials successfully. They are being considered for stomach ulcers due to *Helicobacter pylori* (nisin) and oral mucositis (IB-367) [44].

Derivatives and/or synthetic products of insect defensins could be of high value in agriculture and in public health. Understanding of the structure of this peptide from mosquitoes and thereby its function could help development of highly active peptides that may restrain pathogens invading the vector mosquitoes, thereby preventing vector-borne diseases. Therefore, the results of this study could be of great help in future studies against vector-borne diseases.

Summary

Innate immunity is one the earliest evolved defence mechanisms. It spans through the simplest organisms to the highly complex *Homo sapiens*. Defensins, one of the most important antimicrobial peptides, are widely recorded and have been extensively studied. Although the 3D structure of defensins of higher animals and plants, and many other organisms, have been studied, the structure of defensins from the dengue vector mosquito, *Ae. aegypti* was not known until now. Structural variations among the isoforms from a single individual were also not known. Therefore, the prediction experiment reported here was carried out based on homology of related structures. Prediction was done in Modeller 9v1 and validated using Procheck, total, local, burial and contact energies and RMSd values. The three best models (each per isoform) are presented here.

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