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Potential anticancer peptides design from the cysteine rich plant defensins: An *in silico* approach

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Cancer is the second leading cause of mortality worldwide preceded by cardiovascular diseases. The therapeutic approaches for drug developmentinclude the use of small molecules, antibodies, peptidesor short nucleic acid sequences. The peptide-based drugs have been developed to treat many diseases like cardiovascular diseases, cancer, metabolic disorders, immunological diseases and viral infections. More than 80 peptide drugs are already in the market. These therapeutic peptides have several important benefits over antibodies and proteins due to their small size, ease for chemical synthesis and further the ability to penetrate cell membrane. Furthermore, peptide drugs have high specificity, activity, and affinity. The plant defensins BcDef1, TPP3, NaD1, 2N2R and 2LR3 have been studied for their role in wide range of diseases. This study focussed on the conformation of plant defensins rich in disulfide bonds. The structure for BcDef1 has been predicted from the conformational ensemble. Then, we designed anticancer peptides from these defensins with computational methods. The designed anticancer peptides have been studied for their immunogenicity as well as homology with human proteome. The role of designed peptides has been suggested for interferon-gamma induction, the later has been shown to possess a very important role in cancer.

Keywords: Anticancer peptides, Cancer, Interferon-gamma induction, Peptides, Plant defensins

The living organisms are exposed to a variety of pathogens, which result in several health problems involving different mechanisms. Antimicrobial resistance (AMR) is one among these, whichhas become a critical public health issue. AMR occurs, when microbes develop mechanisms to evade antimicrobials, rendering them ineffective[1]. Bacterial infections, responsible for morbidity and mortality, with increased resistance to Gram-negative and Gram-positive bacteria is a challenge to combat these issues[2]. *Staphylococcus aureus* is a major human pathogen responsible for a wide range of clinical infections *viz.* Bacteraemia, infective endocarditis, osteoarticular and skin infections[3]. The host organisms have developed versatile molecules to escape these devastating effects of infections during evolution and these molecules are present in the single celled systems to highly complex organisms.

The plants also protect their homeostasis or curb the invading pathogens with variety of immunological molecules, called defensins, a class of plant immune system. These are short peptides having 25 – 55 amino acid residues which regulate plant health and protect them from harmful pathogens.Thus, these are included among antimicrobial peptides (AMPs)[4]. The plant defensins are present as a set of positively charged (enriched with Lys and Arg) peptides with disulfide (-S--S-) bonds formed by cysteine residues[5,6]. Further, the peptide defensins of plant origin show antimicrobial activities against the microbes causing various human diseases[7]. The rapid development of resistance to conventional antibiotics, the antimicrobial peptides (AMPs) have emerged as a new class of promising antimicrobial drug candidates[8,9]. The use of AMPs to treat drug resistant bacterial infections has gained interest in the recent years. Since naturally occurring AMPs and their synthetic analogues exhibit broad-spectrum antimicrobial activities and these are often regarded as the next generation of antibiotics[10,11].

Plant based peptides have been reported from *Nicotiana alata* (Tobacco)[12], *Raphanus sativus* (Raddish)[13], *Solanum lycopersicum* (Tomato), *Medicago trunculata* (Barrel medic) which show anti-fungal and bacterial activities[14]. The plant defensin PSD1 suggested to interact with cyclin F, and this interaction inhibits human cancer cell cycle[15]. Defensins and defensin-like peptides are functionally diverse and disrupt microbial membranes, act as ligands for cellular recognition and signalling[16].

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Suppl. Data available on respective page of NOPR

A recent study has reported novel defensin (BcDef1) from a plant used in traditional Thai medicine having antispasmodic activity, which has been named as *Brugmansia x candida*[17]. The BcDef1 has mol. mass of 5.29 kDa. Alkaline isoelectric point showed antimicrobial activity against *S. epidermis* ATCC 12228 strain with MIC15 μM, the defensin possess novel sequence with α-core ($GXCX_{3-5}C$) and γ-core ($GXCX_{3-9}C$) motifs. The defensins mentioned above are rich in Lys, Arg and Pro residues, hence have considered to design the potential anticancer peptides. Keeping this in view, the present study was carried out on these therapeutically potent peptide based drugs, to throw light on their conformation, and in particular the role of cysteine residues in defensins, which may help to design and predict potential anticancer drugs from various plant sources. These results are presented and discussed in this communication.

Materials and Methods

The crystal structure of various plant defensins with accession numbers *viz.* TPP3, 2N2R, 2LR3 and NaD1 available at RCSB except the BcDef1 were used. The amino acid sequence of BcDef1 subjected to the ProtParam server and the molecular weight, pI and charge on the defensin were obtained. The three-dimensional model for BcDef1 was generated using P2v2S server[18]. The disulfide bonds were predicted by disulfide predicting server[19]. The predicted model obtained was further subjected for energy minimization with SPDBviewer[20] to remove the steric clashes between the atoms. Furthermore, the crystal structures were downloaded from RCSB for tomato defensin, *Nicotiana alata* defensin 1, *Medicago truncatula* defensin (MtDef4) and radish defensin with PDB Ids – TPP3, NaD1, 2LR3 and 2NR2, respectively. The solvent molecules were removed from the structures in PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.) and subjected to energy minimization with SPDBviewer. Independent multiple molecular dynamics (MD) simulations for each defensin were performed in SPC water model at 300 K with GROMACS software version 5.1.4[21]. For every system setup, the peptide was kept in the center of a cubic box with 1.0 nm distance from molecular surface. The system was neutralized by adding corresponding ions using *genion* module of GROMACS followed by energy minimization to remove steric clashes between atoms of the peptide and water molecules. Then, the simulation conditions for NVT and NPT were used before each final MD simulation run for 10 ns. The detailed MD simulation procedure used were described earlier[22].

The convergence or the quality of simulations were checked by Cα RMSD, radius of gyration and total energy plots for each simulation. The free energy landscape, for each defensin system, were calculated from concatenated trajectories from multiple simulations. The final representative conformation with lowest energies were extracted and analysed. The GROMACS modules *viz*. *trjconv*, *rms*, *gyrate*, *rmsf*, *energy*, *rama*, *omega* and *cluster* were used for the analysis of MD simulation results for the studied systems of all plant defensins. The data was plotted with xmgrace[23]and the structure analysis was done with PyMOL software .

Results and Discussion

Analysis of simulated trajectories

In order to understand the conformations adopted by the defensin molecules with time and the factors contributing to their stability, we performed equilibrium molecular dynamics simulations on the defensins, BcDef1, NaD1, 2N2R, 2RL3 and TPP3. In our present study we have monitored the role of disulfidebridges, hydrogen bonds and other factors for the conformational stability of defensins.

The analysis of MD simulated trajectories showed that the root mean square deviation (RMSD) values have converged and get stabilized after three nanosecond of simulation time (Suppl. Fig. 1A). The residue specific fluctuations have been observed in terms of root mean square fluctuations. The higher the RMSF values shown by more flexible the regions. The root mean square fluctuations (RMSF in nm) for Cα atoms of residues present in each defensin have revealed that the flutuations occur mainly in the loop regions. The regions like α-helix and β-sheet have less fluctuations. The analysis revealed that the defensins NaD1 and TPP3 have even lesser RMSF values than the other studied defensins *viz*. BcDef1, 2LR3 and 2N2R.

The total energy and radius of gyration (Rg in nm) were also analysed for all the systems considered for MD simulations (Suppl. Figs 2 & 3). The analysis of the plots showed that Rg values are less in NaD1 and TPP3 in all three coordinates as compared to other three defensins, although BcDef1 has less Rg

values among these three systems. The total energy for all systems gets stabilized before 1 nsand the energy for BcDef1, 2LR3 and 2N2R remained within same range, both NaD1, TPP3 have similar energy range.

The defensin BcDef1 have two low energy basins as shown in (Fig. 1), their representative conformations have RMSD difference of 1.602 Å (Suppl. Fig. 3) while RMSD value differed from the starting geometry of 1.931 Å. The maximum difference was observed in the loops namely L1 and L3 (Suppl. Fig. 4).

Free Energy Landscape

The conformational stability of defensins were calculated to obtain the representative stable conformations. The free energy landscapes (FEL), for all systems and the conformations, are shown corresponding to low energy state of the defensins. The RMSD and Rg values have been plotted to calculate the free energy of plant defensins, hence representative conformations for each defensin studied. The BcDef1 defensin have two low energy basins (dark blue coloured regions in 2D FEL plot)

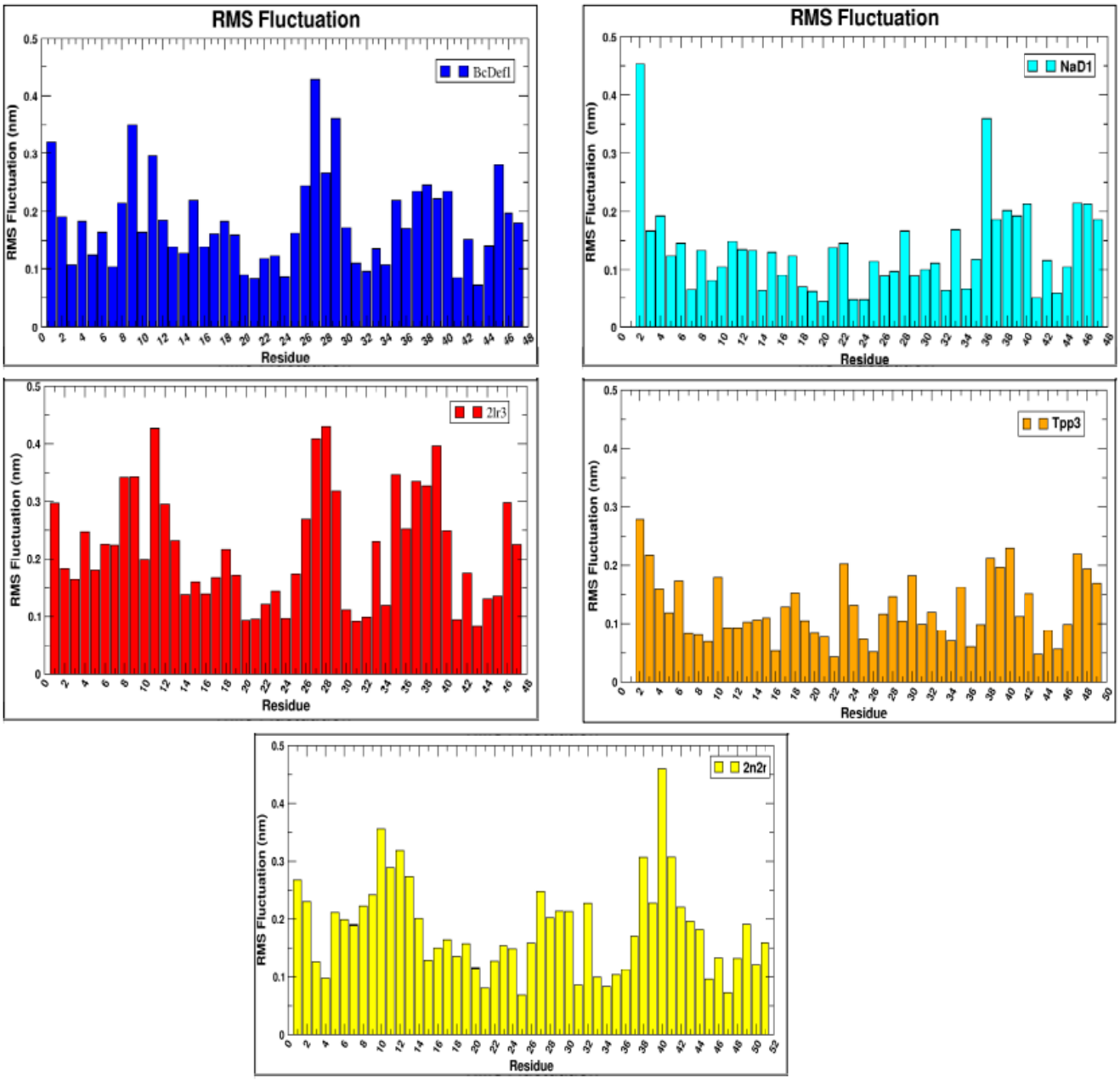

Fig. 1 — The bar diagrams for Cα atom root mean square deviation (RMSF) from molecular dynamics simulations in water

and its corresponding stable conformations have been shown, (Fig. 2). Similarly, the defensin, 2LR3 has two low energy basins while others converge to single one and their respective stable conformations have been shown with cartoon representations in (Fig. 2). The

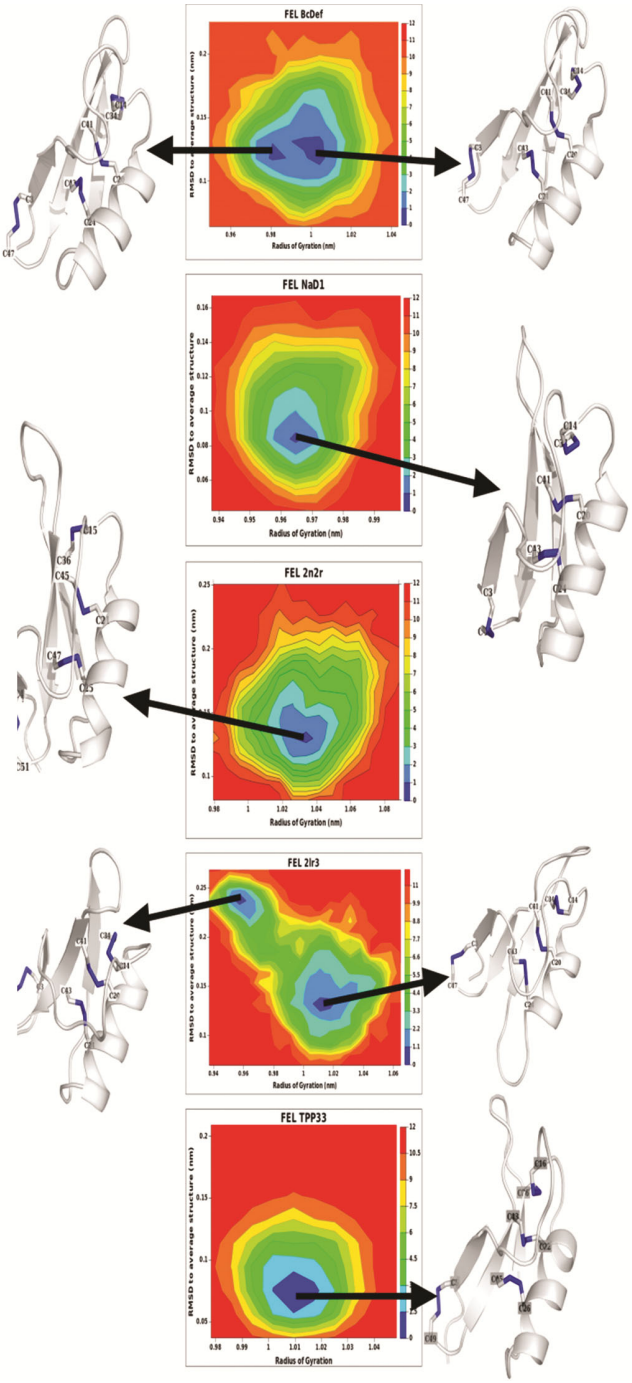

Fig. 2 — The Free Energy landscapes (FEL) for defensin, BcDef1, NaD1, 2N2R, 2L2R andTPP3 (top to bottom) and the representative conformation (stable) obtained from the lowest basins of FEL

major differences were observed in the loop regions, which is furtherevident from the RMSF plots, as shown in (Fig. 1). The φ, ψ values have been plotted for representative conformations of BcDef1, which are found in allowed regions of Ramachandran map.The ω values have been found within 180° ±25 for all the systems (data not shown). The side chain groups of phenylalanine (Phe) residues have been found to protruding away from the main structure *i.e.*, there are no aromatic-aromatic interactions in between the residues, (Fig. 6).

Analysis of the disulfide bonds

The defensins contain conserved cysteine residues, which participate in disulfide bonds and provide stability to the three-dimensional structure. The conserved disulfide bridges (-S--S-) participate in redox reactions; emphasize their biological/physiological importance[24]. The plant defensins, studied herein contain eight cysteine residues, resulting in four disulfide bonds through side chain sulfur atoms. A study on native disulfide bonds reported the average values of the Cβ-S-S′-Cβ′ torsion to be left-handed and right-handed, around −87° and +97°, respectively[25]

The disulfide bonds for BcDef1 were predicted[19], the sequence analysis revealed, it has similar cysteine motif as studied in NaD1, TPP3, 2LR3 and 2N2R defensins, (Table 1). The distributions for disulfide bonds are shown in (figure 3), obtained from simulated trajectories in water of the studied peptides. The Cβ-S-S′-Cβ′ dihedral angle (disulfide bond dihedral) has mainly two types of symmetry; positive dihedrals, 60° to 120° / negative dihedral values, −60° to −120°. The chi-3 dihedral (Cβ-S-S′-Cβ′) distributions plots shown in (Fig. 3) for the studied peptides: BcDef1 has 2 negative (first and third), 2 positive (second and fourth) values, similar type of behaviour have been observed for 2lr3 peptide. The disulfide bond dihedrals in tomato defensin (tpp3) showed opposite behaviour *i.e.*, first & third have positive values while second & fourth have negative values distributions.

The NaD1 defensin have Cβ-S-S′-Cβ′ dihedral values with first, second and fourth in negative

Table 1 — The defensin residues predicted to involve in disulfide (-S--S-) bonds

Disulfide Bond	BcDef1	NaD1	2LR3	TPP3	2N2R
1	3, 47	2, 46	3, 47	4, 48	4, 51
2	14, 34	13, 33	14, 34	15, 35	15, 36
3	20, 41	19, 40	20, 41	21, 42	21, 45
4	24, 43	23, 42	24, 43	25, 44	25, 47

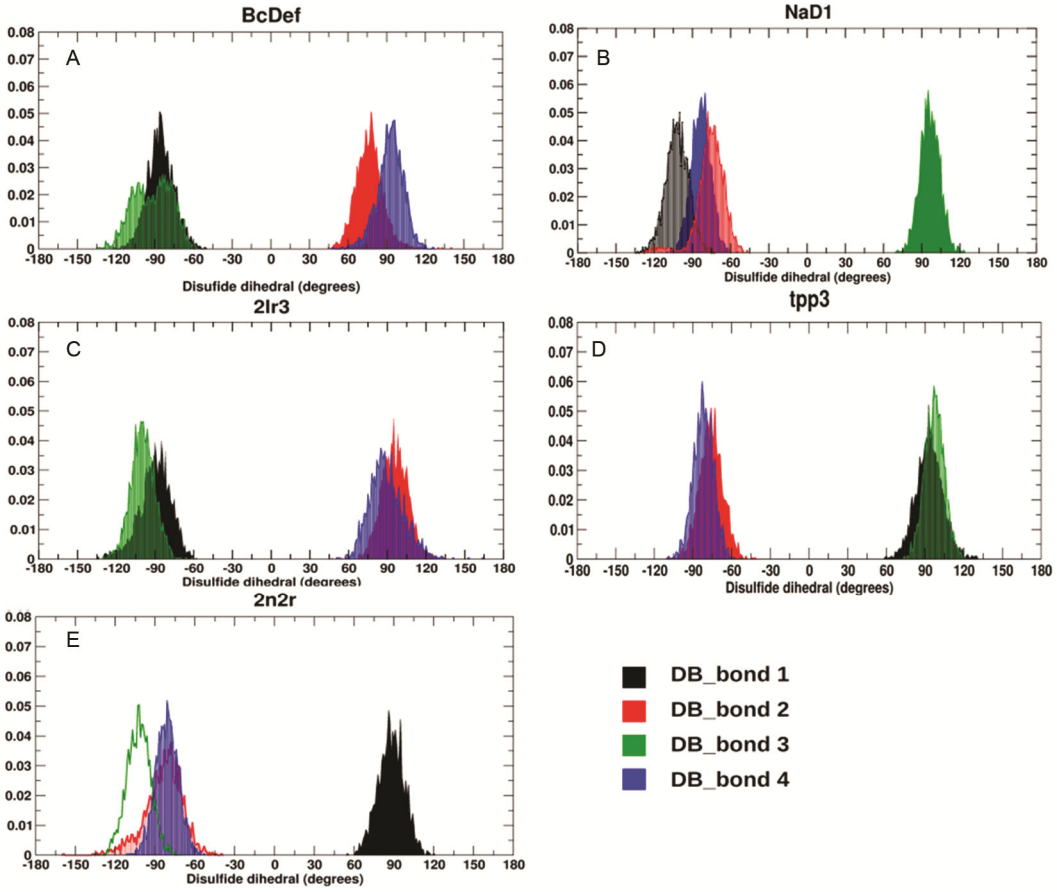

Fig. 3 — The distributions for disulfide dihedrals (Cβ-S-S′-Cβ′) of defensin molecules studied in in water at 300 K. The corresponding colour is defined for the distribution (DB = disulfide bond)

region, while third distribution lies in the positive region. Similarly, the 2N2R defensin have first Cβ-S-S′-Cβ′ dihedral in positive region, the others three (2, 3 & 4) restricted in the negative region during MD simulations. The dihedral angles for disulfide bridges, have been given in (Suppl. Table 1), corresponding to each defensin for most stable conformation.

The secondary structure analysis

The number of residues present in regular secondary structure is an indicator of the stability of the defensin. The trajectories obtained for each defensin have been analysed for secondary structure elements, pronounce during simulation time. The plots showed that the α-helices are the most stable secondary structures in all the defensins studied (Suppl. Fig. 1B). The β-sheets of BcDef1 and tpp3 are more stable followed by NaD1, 2LR3 and 2N2R peptides. The fluctuations in the other secondary elements vary during the time evolution of the peptides as these can also be observed fromCα RMSF of the residues as depicted in (Fig. 1). The representative conformation for each system was subjected to PDBSum server for secondary structure analysis, it was plotted as line for a coil, spring for an α-helix and an arrow for β-sheet[26].

The circular dichroism spectroscopy of proteins is very sensitive and informative technique. It requires subtle amount of protein/peptide to predict the secondary structure of proteins[27]. There are a number of computational tools available to calculate the CD spectra from molecular dynamics ensemble or average structures[28,29]. The secondary structure elements were verified using PDB2CD spectrum predicting server for all the representative structures of the studied defensins. The structure with lowest energy for each defensin was submitted to the server to predict CD spectrum, which have shown in (Fig. 4).

The predicted spectral results are supported by the PDBSum results for each resultant secondary structure of defensin after simulations, (Fig. 5). The Supplementary Table 2 depicts that the defensin

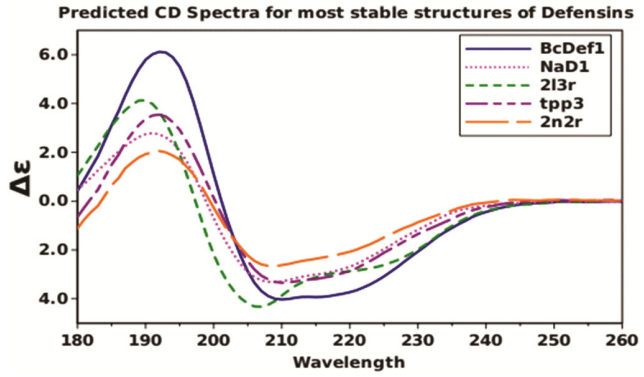

Fig. 4 — The Circular dichroism results for the most stable conformations obtained lowest energy basin (FEL) for each defensin *viz.* BcDef1, NaD1, 2l3r, tpp3 and 2n2r

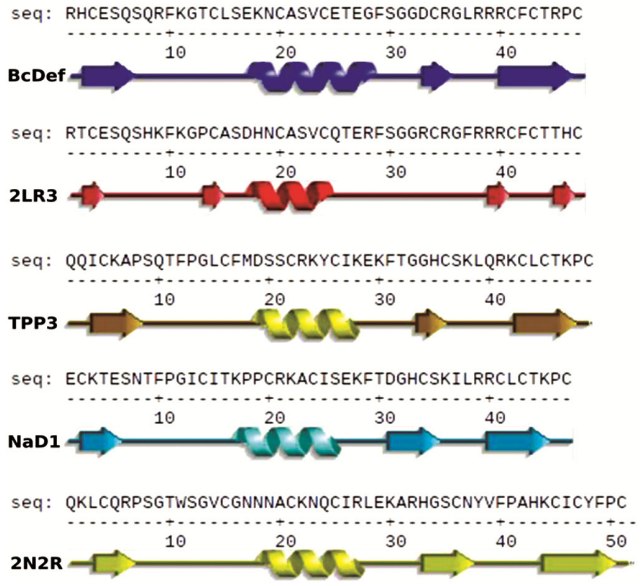

Fig. 5 — The secondary structure for BcDef1, 2l3r, tpp3, NaD1 and 2n2r defensins. The arrows, springs and lines encode strand, helix and turns/loops, respectively

BcDef1 has helix, sheet and other structures as 28.4%, 29.1% and 46.1%, respectively. Furthermore, the helical content is present in increasing order 2L3R (17%) <2N2R (18.93) <TPP3 (21.5) and NaD1 (22.43) defensin. The sheet is highest in NaD1 and least in 2l3r while the secondary structure elements other than helix and sheets are found to be highest in 2l3r and least in NaD1 defensin. Similarly, the CD spectra predictions, (Fig. 4), supported the PDBSum results as discussed above.

Analysis conserved sequence

The plant defensins being diverse but they have only eight conserved residues *viz*. cysteine (C), two glycine (G) and one aromatic residues (phenylalanine; F, tyrosine; Y and tryptophan; W). The careful analysis of the studied defensins revealed that they have phenylalanineresidues, but all are projected outwards. The presence of residues like tyrosine, phenylalanine or proline suggested to increase the cytotoxic activity of the synthesized peptides[30]. Further, the secondary structure of anticancer peptides plays a crucial role in peptide-membrane of cancer cell interaction, leads to cancer cell disruption and death[31]. The Phe residues play an important role in lysis of bacterial membranes and have least haemolytic activity[32].

The structure analysis of defensins under reference revealed that the phenylalanine residues are protruding from the core of the peptides, (Fig. 6). These results suggested that the Phe residues have more potential to interact with membrane due hydrophobic interactions between side chain of the residue and lipids of outer leaflet. The cancer cell membranes possess negative charge on their surface that can be countered by peptides containing positively charged residues, lysine and arginine. The structure analysis of defensins showed that all the peptides have net positive charge at physiological pH. The orientation of side chain of Phe residues in all studied defensins is protruding from the core structure shown in corresponding Figure, which provides the hydrophobic part (side chain of Phe residues) to interact with the hydrophobic membrane of the cells.

Defensins are also reported to be active against various cancer cell lines. Furthermore, the peptide based anticancer bioactive molecules are at various phases of drug development[33]. Recently, US-Food and Drug Administration approved therapeutic peptides, proteins and their 380 drug variants[34].

The sequence analysis of the studied defensin peptides using AntiCP server have predicted potential anticancer peptides (Suppl. Table 3). The predicted potential anticancer peptides *viz. FKGTCLSEKN*, *CRGLRRRCFC* (BcDef1); *CRGFRRRCFC*, *RRRCFCTTHC* (2L2R); *KPPCRKACIS* (NaD1); *DSSCRKYCIK* (TPP3); *LEKARHGSCN* (2N2R) were further designed to increase their anticancer potential (Table2). The selection of these peptides was based on the highest support vector machine score (SVM Score) obtained from the server[35]. The mutated peptides possess replacementwith cysteine or a charged residue. The change of amino acid residue with cysteine prefers disulfide bonding and hence increases stability of peptide structure. The peptide drugs can generate

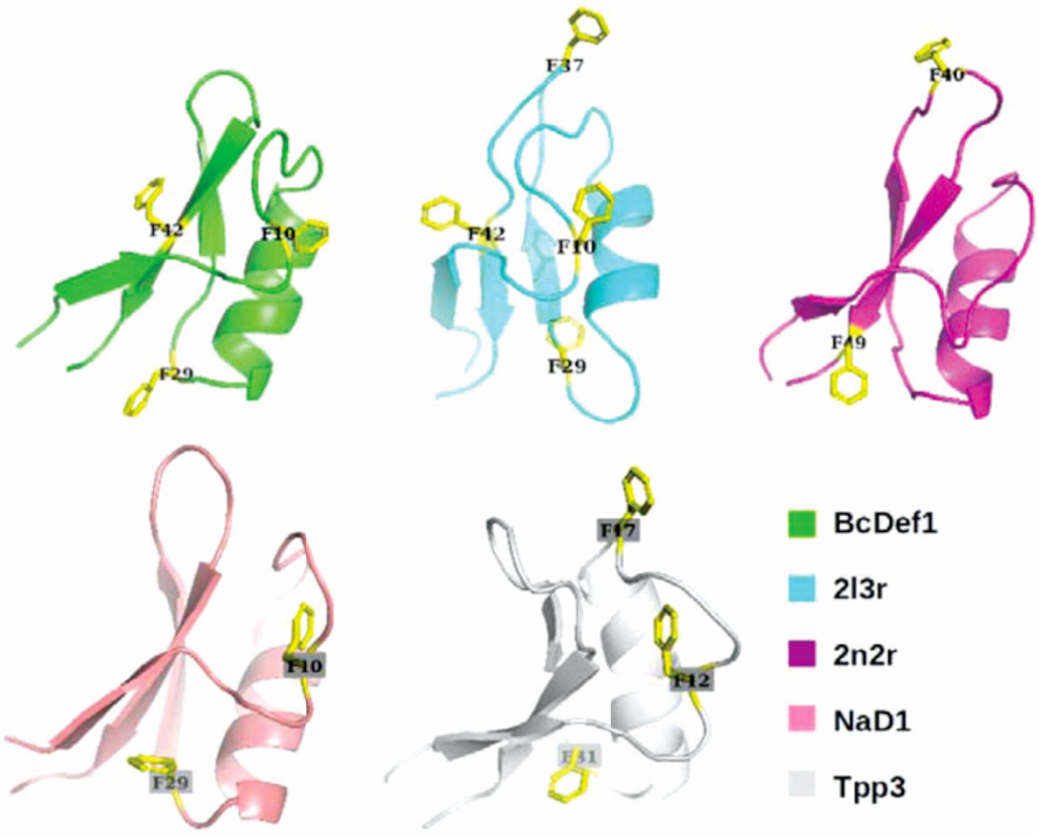

Fig. 6 —The cartoon representation of the defensins with Phe residues at the active/interacting/loop regions shown in yellow sticks

Table 2 — The predicted anticancer peptides and their derivatives with increased anticancer potential, binding affinity to HLA I alleles.

Defensin	Predicted anti-cancer peptide (PACP)	Mutated anti-cancer peptide (MACP)	MHC I allele	Affinity (nm)	Bind Level	IFN- γ induction
BcDef1	FKGTCLSEKN	FKGTCLSEKK	*None*	>3000	NB	+
	CRGLRRRCFC	CRGLRRCCFC	HLA-B0801	145	SB	+
2L2R	CRGFRRRCFC	CRGFRRCCFC	HLA-B0801	189	SB	+
	RRRCFCTTHC	RRRCFCTEHC	HLA-B2705	382	WB	+
NaD1	KPPCRKACIS	KPPCRKACIC	HLA-B0702	1096	WB	+
		KPPCRKACIK	HLA-B0702	1096	WB	+
TPP3	DSSCRKYCIK	DSSCREYCIK	*None*	>3000	NB	+
2N2R	LEKARHGSCN	LEKCRHGSCN	*None*	>3000	NB	+
		LEKARHGSCK	*None*	>3000	NB	+
		FEKARHGSCN	HLA-B0801	693.99	WB	+

immunogenicity to humans, hence the sequence similarity with human proteome has been cross validated with protein blast tool from NCBI. Recently, the designed potential peptides therapeutics have been subjected to check the immunogenicity using the protein blast tool[36].

IFN gamma prediction

Interferon-γ (Ifn-γ) plays a key role in activation of cellular immunity and subsequently, stimulation of antitumor immune-response. The peptide sequences were screened for interferon-γ prediction[37] and epitope design. The mutated anticancer peptides predicted to be promiscuous for the induction of interferon – γ. The cytokine Ifn- γ has very important role in antitumor immunity. The strong binding peptides to MHC I alleles from BcDef and 2L2R (CRGLRRCCFC, CRGLRRCCFC, respectively) further showed the promising results to induce anticancer immunity (induction of Interferon- γ). The second important role of interferon- γ has been suggested for antiviral and antitubacterial, especially in tuberculosis[38] as interferon is the key mediator for macrophage activation[39].

These results suggest that the experimental studies must be carried out for therapeutic developments in cancer therapy. The promiscuous peptides sequences CRGLRRCCFC and CRGFRRCCFC against antitumour immunity may provide promising results to combat the second deadliest killer of humans, the cancer followed by cardiovascular diseases[40]. The selected peptides were further worked for increasing the binding affinity and anticancer property and we found the CRGLRRCCFC and CRGLRRCCFCN peptides as more potential candidate possessing anticancer property.

Conclusion

The present study gives an insight to the design and prediction of antitumour activity of plant defensin derived potential peptide therapeutics. The designed mutated anticancer peptides (MACPs) CRGLRRCCFC and CRGLRRCCFCN have shown the induction of Ifn-γ, which have been suggested to play a very important role in antitumour immunity. The peptide based therapies imparting better effects on the immune system as compare to traditional drugs[41]. The antimicrobial resistance by various pathogens create obstacles to combat complicated diseases like cancer and immuno-compromised conditions[42,43]. It is suggested that these MACP peptides must have a considerable potential to develop the anticancer therapeutics. These can be considered for experimental studies against cancer cell lines followed by animal models. Recently, the peptide based therapeutics have been designed and thier role in induction of Ifn-γ have been suggested[36]. The interferon- γ is a pleiotropic molecule, is associated with the anticancer immunity[44], it is secreted by CD8 cytotoxic T cells[45], antigen presenting cells[46] and natural killer cells[47], which all participate in tumor evasion.

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Conflict of interest

All authors declare no conflict of interest.

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