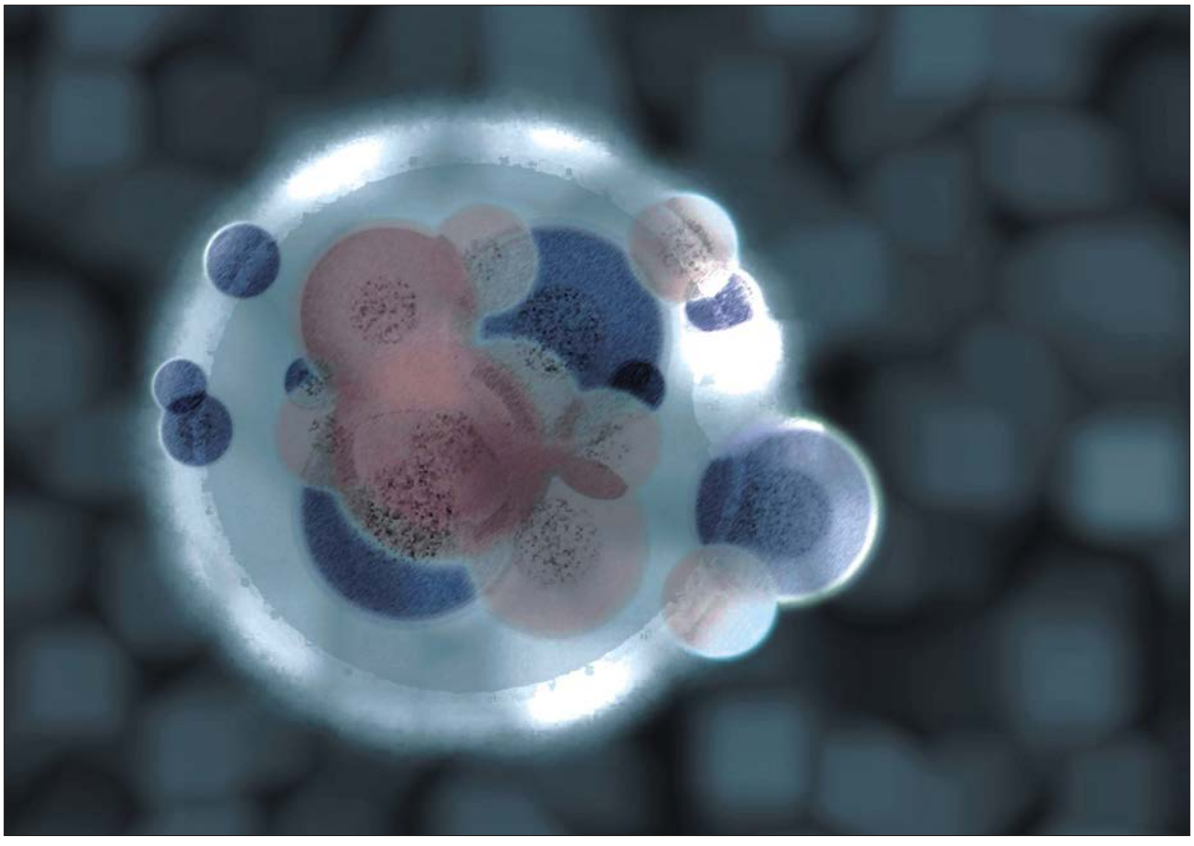

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Evaluation of Structural Features of Membrane Acting Antifungal Peptides by Artificial Neural Network

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Abstract: This study is aimed at determining the importance of structural descriptors on bioactivity of antifungal peptides by ANN methodology. Due to vital need for novel fungicidal agents, understanding structural features of antifungal peptides and their mechanism of action is necessary. The main AFP actions are interaction with fungal membrane components, formation of membrane channels and pores and eventually fungal cell membrane degradation. Since these mechanisms depend on structural features of the peptides, study of the relationship among these features can give new insights into mode of action of antifungal peptides. In this study, Artificial Neural Network (ANN) is used to determine the key parameters in bioactivity of antifungal peptides. Among a set of 12 molecular descriptor for nearly 60 naturally originated antifungal peptide, α-helical content, hydrophobic interaction and charge positivity introduced as the most important parameters in AFPs activity. This result confirm the barrel-stave pore model (BSPM) as the most probable action mechanism of antifungal peptides.

Key words: Antifungal peptide, structural descriptor, evaluation, artificial neural network, BSPM

INTRODUCTION

Antimicrobial peptides (AMPs) are short peptides (<100 amino acids), mainly containing α-helical structure with a potent function against microbial invasion. These peptides are essential components of innate host defense in species with a wide range of diversity (Andreu and Rivas, 1998). These peptides are very diverse with respect to amino acid sequence and secondary structure but share certain properties, such as affinity for the negatively charged phospholipids that are present on the outer surfaces of the cytoplasmic membrane of many microbial species. These peptides are present as ubiquitous, simple and effective agents acting within the innate immune system. Their short length and fast and efficient action against microbes has made them potential candidates as drugs (Van't Hof *et al*., 2001; Loffet, 2002). Several peptides and their derivatives have already passed clinical trials successfully and several others are considered as potential therapeutics (Hancock and Chappel, 1999; Levy, 2000). Other than having pathogen-lytic activities, these peptides have other properties like anti-tumor activity, mitogen activity, or act as signaling molecules (Kamysz *et al*., 2003). Moreover, they have a number of biotechnological applications, e.g. in transgenic plants (Baker *et al*., 1997), in aquaculture and as aerosol spray for patients of cystic fibrosis (Osusky *et al*., 2000). Among several mechanisms that have been suggested for AMPs' activity, the three most cited are Barrel-Stave Pore Model (BSPM), Toroidal Pore Model (TPM) and the carpet mechanism. According to BSPM, AFPs should have distinct structure such as α-helix or β-sheet or both and have hydrophobic interaction with the target membrane (Selitrennikoff, 2001). On the other hand, TPM is based on formation of several short-lived clusters of an undefined nature of secondary structure (Yeman and Yount, 2003). The carpet mechanism is nothing but repetition of TPM, i.e., AMP molecules carpet the surface of a target membrane and when sufficiently accumulated, create numerous toroidal pores (Oren and Shai, 1998) (Fig. 1). Among AMPs, there is a group with considerable fungicidal effect referred as antifungal peptides (AFPs). Due to high losses arise from fungal invasions, increasing attempts are made to isolate, synthesize or improve the performance of AFPs. These attempts need vast knowledge about AFPs' structure and mechanism of action. Better understanding of the structural and functional features of AFPs has led to peptide derivatives with higher affinity to fungal membranes and increased fungitoxicity as a result. Recently, significant progress has been made in use of Computer-Aided Molecular Design (CAMD) of novel molecules with desired properties, these methods typically rely on two stages: the first stage is forward modeling, through which

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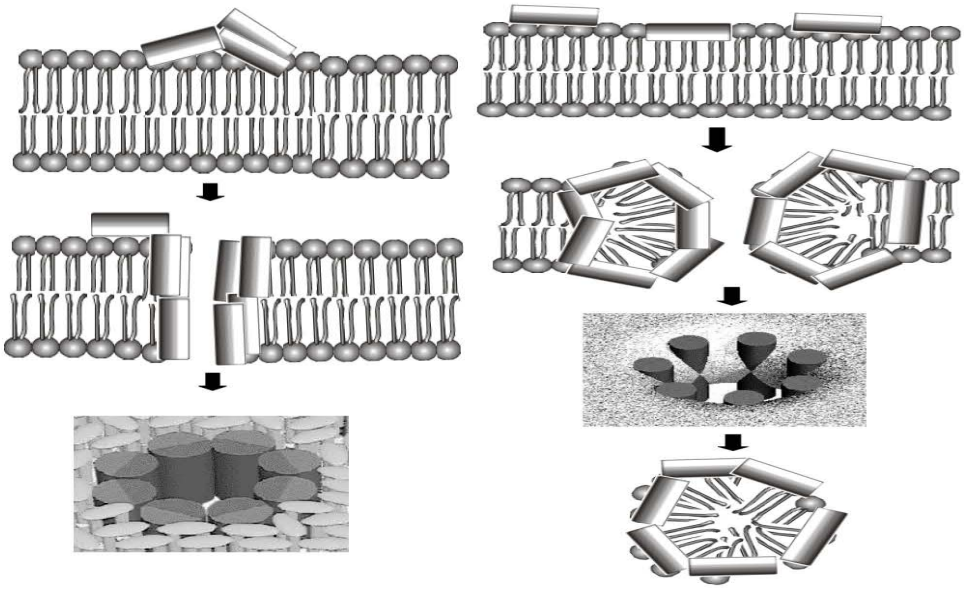

Fig. 1: This figure illustrating the barrel-stave pore model (BSPM) (left) and the carpet model (CM) (right) presented for membrane permeation. In the barrel-stave model, peptides first assemble on the surface of the membrane then insert into the lipid core of the membrane following recruitment of additional monomers, forming pores. In the carpet model, the peptides are bound to the surface of the membrane with their hydrophobic surfaces facing the membrane and their hydrophilic surfaces facing the solvent. When a threshold concentration of peptide monomers is reached, the membrane is permeated and transient pores can be formed, a process that can lead also to membrane disintegration

Quantitative Structure Activity Relationship (QSAR) process is accomplished by application of non-linear modeling procedures such as Artificial Neural Networks (ANN) and the second stage is model inversion/optimization which is the use of optimization algorithms in exploitation of the first stage results in discovery of molecules with improved activity (Patel *et al*., 1998). Several parameters affect the activity of antifungal peptides such as sequence, size, charge, degree of structure formation, cationicity, hydrophobicity and amphipathicity. Use of ANN could help us evaluate the importance of these structural parameters in bioactivity of AFPs and eventually the most probable model of AFPs' mechanism of action. The antimicrobial peptides have little sequence homology, despite common properties (Loffet, 2002). Thus it is difficult to develop a specific method for predicting the antimicrobial peptides based on similarity. Moreover, experimental methods for identification and designing of antimicrobial peptides are costly, time consuming and labor-intensive. Thus there is a need to develop computational tools for predicting antifungal peptides, which could be used to design potent peptides against fungal pathogens. Recently attempt is done to model antimicrobial peptide by QSAR and other *in silico* methods. For example, a HMM (Hidden Markov Model) based method has been developed for searching conserved motifs of β-defensin family in genome databases (Scheetz *et al*., 2002). QSAR bioactivity modeling of antimicrobial dodeca peptide of Bac2A indicated that good activity was not only dependent on the composition of amino acids or the overall charge or hydrophobicity, but rather required particular linear sequence patterns (Hilpert *et al*., 2006). In a study, the construction of a mathematical model for prediction, prior to synthesis, of peptide antibacterial activity toward *Pseudomonas aeruginosa* has been described (Jenssen *et al*., 2007a, b). By use of novel descriptors quantifying the contact energy between neighboring amino acids in addition to a set of inductive and conventional quantitative structure-activity relationship descriptors, it is possible to model the peptides antibacterial activity (Jenssen *et al*., 2007a, b). Furthermore, by use of smoothed amino acid sequence descriptors, the structural characteristics important for antimicrobial activity are determined (Fernandez *et al*., 2007). In the present study, a systematic investigation has been made to better understand the importance of this class of peptides and to develop an algorithm for predicting antifungal peptides with high accuracy.

MATERIALS AND METHODS

The information about the peptides was extracted from the published scientific papers as well as related websites (Table 1). Major databases on antimicrobial peptides are:

I) ANTIMIC have around 1700 sequences (Brahmachary *et al*., 2004) II) AMSDB consists of 804 antimicrobial peptides of eukaryotic origin (Antimicrobial Sequence database [http://www.bbcm.units.it/~tossi/amsdb.html]. The database was originally created as part of the thesis work of undergraduate students, under the supervision of Dr. Alex Tossi. More recently it has been updated and maintained within the framework of the European "PANAD" (Peptides As Novel Antiinfective Drugs). III) Peptaibol consists of around 300 antibiotic peptides originated from fungal organisms (Whitmore *et al*., 2004) and IV) APD consists of detailed information for 525 antimicrobial peptides (Wang Wallace and Wang, 2004).

According to the above-mentioned mechanisms of action, a peptide which has lethality effect on fungi should be capable of forming ion channels in membrane by aggregation (pseudoionophores) and insertion in order to span cell membrane with 2.5-4.0 nm thickness (depending upon lipid composition) and should have at least 15 amino acids residue (Giangasparo *et al*., 2001). Furthermore the peptide should be alpha-helical to pass through the membrane (Eunoh *et al*., 1999). Being laterally amphipathic (one face of the helix displaying hydrophobic residues, while the opposite face displays hydrophilic residues), the peptide can form hydrophilic ion channels or pores and at the same time remain in contact with the hydrophobic components, e.g. fatty acyl moieties. These structural features can be mentioned using several structural descriptors (Shai, 2002). It should be mentioned here, that some structural descriptors may reflect related or similar molecular/atomic properties and can be correlated in certain cases, even though the analytical representations of those descriptors do not directly imply their co-linearity. Therefore, special precautions were taken in selecting the appropriate structural descriptors for the QSAR model. Hence, to eliminate the cross-correlation among the independent variables, we pre-computed pairwise regressions between all pairs of the descriptors using NCSS (Hintze, 1992) software. We subsequently removed those descriptors that linearly correlated with $R \geq 0.9$. As a result of this procedure, only 12 parameters were selected.

1: Length: Number of peptide residues. Results from previous analyses about length effect in the anti-infective potency of AMPs indicate contradictory results.

Table 1: The antifungal peptides according to their year of discovery and length

Name	Length	References	Name	Length	References
Mycocerein	16	Wakayama *et al*. (1984)	Ginkbilobin	40	Wang and Wang (2000)
Drosomycin	44	Michaut *et al*. (1996)	GAFP	38	Wang and Wang (2004)
Nostofungicidine	7	Kajiyama *et al*. (1998)	Gaegurin	25	Wang and Wang (2004)
Anafp	55	Lee *et al*. (1999)	Ponericine G1	30	Wang and Wang (2004)
SMAP-29	28	Skerlavaj *et al*. (1999)	Spingerin	25	Wang and Wang (2004)
MP	11	Eunoh *et al*. (1999)	Termicin	36	Wang and Wang (2004)
Ranatuerin-2	37	Rozek *et al*. (2000)	Pseudin1	24	Wang and Wang (2004)
IWF6	37	Kristensen *et al*. (2000)	Nigrocin 2	21	Wang and Wang (2004)
Brevinin-2	34	Ng (2000)	Pinin	45	Wang and Wang (2004)
DSP	41	Gao *et al*. (2001)	Heliomicin	44	Wang and Wang (2004)
PAFP-s	38	Yang *et al*. (2002a, b)	EAFP1	41	Wang and Wang (2004)
Cicadin	54	Wang and Ng (2002)	Hepcidin20	32	Wang and Wang (2004)
Tritrpticin	13	Yang *et al*. (2002)	Eryngin	90	Ng and Wang (2004)
DRR230-c	33	Lai *et al*. (2002)	Ucurmoschin	73	Wang and Wang (2004)
Histatin	43	Banzet *et al*. (2002)	Nonapeptide	9	Wang and Wang (2004)
Pe-AFP1	23	Huang *et al*. (2002)	Nartazin	64	Wang and Wang (2004)
Rs-AFP2	51	http:/www.uclan.ac.uk/facs/science/biology/bru/amp_data.htm.	Coccinin	63	Ngai and Ng (2004)
Gymnin	26	Wong and Ng (2003)	Di-K19Hc	19	Wang and Wang (2004)
Cicerarin	20	Chu *et al*. (2003)	Tenecin 1	44	Wang and Wang (2004)
Basrubrins	40	Tanaka *et al*. (2003)	PMAP-23	23	Kumar *et al*. (2005)
Dermaseptin	34	Wang and Wang (2004)	Isarfelin	13	Guo *et al*. (2005)
WGA	42	Wang and Wang (2004)	Agrocybin	15	Ngai *et al*. (2005)
MBP-1	33	Wang and Wang (2004)	Coconutin	90	Wang and Ng (2005)
Esculentin-1	46	Conlon *et al*. (2004)	Vulgarinin	63	Ng and Wong, (2005)
AC-AMP2	30	Wang and Wang (2004)	Callipeltins F-I	34	Sepe *et al*. (2006)
Buforin I	39	Wang and Wang (2004)	Ib-AMP1	20	Merchant *et al*. (2006)
BMAP-27	27	Wang and Wang (2004)	Brevinin1BYa	24	Pal *et al*. (2006)
Pn-AMP2	40	Wang and Wang (2004)	β-purothionin	40	Oard and Karki (2006)
Pn-AMP1	41	Wang and Wang (2004)	Halocidin	20	Jang *et al*. (2006a)
Fn/23	26	Klotz *et al*. (2004)	Tat	11	Jung *et al*. (2006b)

Scott *et al.* (1999) in a study on the biological properties of structurally related α-helical cationic AMPs, designed a series of α-helical cationic antimicrobial peptide variants with small amino acid changes. Alterations in the length of the variant peptides did not improved the antimicrobial activity and there was no statistically significant correlation between this factor and the MIC value for *Pseudomonas aeruginosa*, *Escherichia coli* or *Salmonella typhimurium* (Scott and Hancock, 2000). To evaluate the effect of chain length on antimicrobial activity, Liu *et al.* (2007) synthesized a series of peptides containing simple sequence repeats, (RW)n-NH_2 (n = 1, 2, 3, 4 and 5) and determined their antimicrobial and hemolytic activity. The antimicrobial activity of the peptides increased with chain length and so did the hemolysis of red blood cells. Also some of previous studies showed that there is no significant correlation between molecular weight of peptides and IC_{50} values (Raraport and Shai, 1991).

2: Isoelectric point (pI): The pH at which a molecule or surface carries no net electrical charge. This parameter calculated by ProtParam tool (http://expasy.org/tools/protparam.html). It has previously been shown that with decrease in pI of antifungal peptide (targeted against *C. albicans*) down to 6.25, IC_{50} shows a slight and gradual increase; however, antifungal peptides active against plant phatogenic fungi are more active at pI = 5.5 and their activity at pI = 6.5 reaches a plateau (Soltani *et al.*, 2007).

3: Alpha-helix content: Percent of predicted alpha-helix in peptides based on Double Prediction Method (DPM) using Genamics Expression (1.100, 2000) software. Cationic antimicrobial peptides are generally categorized into four structural classes, i.e., α-helical, β-sheet, loop, or extended structures (Boman, 1995). However, there are many peptides that do not fit into this simplified classification scheme. Many bacterially produced peptides, for instance, have two domains, one of which is α-helical in nature while the other has a β-structure. For many peptides these secondary structures are observed only when the peptides interact with membranes; e.g., bovine neutrophil indolicidin is unstructured in an aqueous environment but adopts a boat-like conformation when interacting with membranes and membrane mimetics such as sodium dodecyl sulfate and dodecyl phosphocholine (Rozek *et al.*, 2000). The plasticity of the secondary structure of indolicidin has been suggested to permit different interactions with different molecules, including DNA and membranes (Hsu *et al.*, 2005).

4: Beta turn content: Percent of predicted beta turn in peptides based on DPM using Genamics Expression (1.100, 2000) software. Cationic peptides have been known to alter their flexible secondary structures (Besson and Michell, 1987). Beta turns are found less than other secondary structures in peptides. Previously, it has been shown that approximately 8% of antifungal peptide secondary structures is composed of beta turn (Soltani *et al.*, 2007).

5: Extended strand content: Percent of predicted extended strand content in peptides based on DPM was calculated using Genamics Expression (1.100, 2000) software. The importance of this factor has been shown before. For example, by changing the membrane-associated shape of indolicidin so that the N and C termini were drawn closer together, (and more extended strand structure formed) the activity against gram-negative bacteria was increased (Rozek *et al.*, 2003).

6: Random coil content: Percent of predicted random coil in peptides based on DPM was calculated using Genamics Expression (1.100, 2000) software. Previous NMR and structure studies showed that the peptides have significant populations of essentially random coil conformations in aqueous solution (Vizioli and Salzet, 2002). Furthermore, it has been shown that most abundant secondary structure founded in AFPs are random coils (Soltani *et al.*, 2007).

7: Relative amphipathicity (μH/μH max): Antimicrobial peptides differ remarkably in the length, sequence and structure, but share two common traits in that they are generally polycationic and their active structures are normally amphipathic (Tossi *et al.*, 1997). It seems likely that most AMPs, by virtue of their amphipathic character, will act as detergents at sufficiently high concentrations (Sato and Feix, 2006). The defined ranges of peptide hydrophobicity and sequential amphiphilicity are key requirements for therapeutic effectiveness (Kondejewski *et al.*, 2002). Dennison *et al.* (2005) produced a hydrophathy plot analysis of some AMP peptide sequences to show that there is a progressive increase in hydrophobicity along the primary structure of some AMPs, such as aurein which runs from C→N (Dennison *et al.*, 2005). In contrast, for some other AMPs, there is a progressive increase in hydrophobicity along the primary structure of Citropin, which runs from N→C. Also, it has been shown that for all groups of AMPs, low values of mean hydrophobicity of the segment (<H>) and hydrophobicity tend to correspond to high MIC values and vice versa; however, these tendencies were not statistically significant ($p>0.05$) (Dennison *et al.*, 2005). Previous studies showed that, whilst not statistically

significant, high values of relative amphipathicities (<μH>) tended to correspond to high MIC values and vice versa for the groups of peptides. This trend is the inverse of that described above for (<H>) and implies that there may be an inverse correlation between <μH> and <H> for α-AMPs (Shai and Oren, 2001). By determining the mean hydrophobic moments (μH) using the Eisenberg equation the relative amphipathicity of AFPs were also estimated:

$$\mu H = \left\{ \left[\sum_{n=1}^{N} H_n . \sin \delta \right]^2 + \left[\sum_{n=1}^{N} H_n . \cos \delta \right]^2 \right\}$$

where, Hn is the hydrophobicity index value of residue n and δ(the angle in radians at which successive side chains emerge from the backbone) =100 for peptides in helical conformation. Other than control calculation, the hydrophobicities used in this computation were from the consensus scale of Eisenberg *et al.* (1984). These values are as follows: Ile, 0.73; Phe, 0.61; Val, 0.54; Leu, 0.53; Trp, 0.37; Met, 0.26; Ala, 0.25; Gly, 0.16; Cys, 0.04; Tyr, 0.02; Pro, -0.07; Thr, -0.18; Ser, -0.26; His, -0.4; Glu, -0.62; Asn, -0.64; Gln, -0.69; Asp, -0.72; Lys, -1.10; Arg, -1.76. To facilitate comparisons, the amphipathicity of peptides is given relative to the maximum possible value (μHmax) resulting from a perfectly amphipathic, 18-residue peptide composed only of Ile and Asp, which would thus be assigned a μH/μH max value of 1. For peptides unlikely to assume a helical conformation, this value is hypothetical.

8: Number of positive charge at pH = 7: AFPs Charge plays a crucial role in the activity of AMPs and is a result of the peptides' mechanism of action. Even trivial alterations in peptide charge could make a great difference in the biological activity of AMPs (Lee *et al.*, 1999). In an effort to optimize the antimicrobial activity of magainin peptides by the modification of charge, it was noted that magainin II analogues with cationic charges in the range of +3 to +7 show an optimized antimicrobial activity and selectivity with increased peptide charge up to a threshold value of +5 in association with appropriate hydrophobic properties. However, a charge increase beyond +5, with retention of other structural motifs, led to a dramatic increase of hemolytic activity and a reduction of antimicrobial selectivity by Scott and Hancock (2000). Using PROTEIN CALCULATOR (v3.3, 2006) the number of positive charge at pH=7 was estimated.

9: The sum of number of amino acids between two adjacent positive charges at pH = 5: It is clear that cationic peptides must have a rather high amount of amino acids with a positive charge, such as lysine, histidine and arginine, but of more importance is to find if amino acids with the positive charge have a specific pattern of distribution through the cationic peptides. It has been found that anionic residues were relatively scarce in antimicrobial peptides presumably to maximize cationicity and when present, tended to be in positions that are i±3 or i±4 relative to basic residues (Dennison *et al.*, 2005). Positions 7 and 14 in N-terminal segment of antimicrobial peptides are key sites in determining hydrophobicity and degree of structure, by the fact that their change affects the balance between antimicrobial potency and cytotoxicity. Position 7 can be modulated to tune selectivity, whereas position 14 appears to be less tolerant (Zelezetsky *et al.*, 2005). Descriptor (9) is defined as a number that shows total number of amino acids between two adjacent positive charges at pH 5 in peptide stretch. This parameter calculated by charge distribution analysis using SAPS (http://www.isrec.isb-sib.ch/ software/SAPS_form.html).

10: Hydrophaty: Hydrophaty scale that combines hydrophobicity and hydrophilicity of amino acids' R-group, can be used to measure the tendency of an amino acid to seek an aqueous (negative values) or a hydrophobic environment (positive values). Hydrophaty is ranging from -4.5 for arginine to +4.5 for isoleucine in Kyte and Doolitte index for hydrophaty (Kyte and Doolittle, 1982). Increasing hydrophaty results in a significant increase in the permeabilizing and hemolytic activities of model peptides against target membranes. It is suggested that hydrophaty was more important than hydrophobicity or β-helical content in governing antimicrobial peptide activity (Pathak *et al.*, 1995).

11: Log P: Logarithm of n-octanol/water partition coefficient calculated by ACD/Chemsketch (10.0, 2005). Log P is an important factor affecting the physicochemical properties of bio-active compounds (Otvos, 2005).

12: The number generated by placing sum of amino acids between positive amino acids sequentially along each other at pH = 7: From previous study, it appeared that specific pattern of positive charge distribution needed for antifungal activity is important (Soltani *et al.*, 2007). By charge distribution analysis using SAPS (http://www.isrec.isb-sib.ch/software/SAPS_form.html) this parameter was calculated.

NEURAL NETWORK

The ANN procedure, a multilayer perceptron with optimized weights, was accomplished by Easy NN-plus 8.01 (1999-2001). The first layer of nodes, which receive the input, is called input layer. The layer of nodes producing the output values is called the output layer. Layers of nodes between the input and output layers are referred to as hidden layers (Lehrach *et al.*,

Table 2: The neural network architectures produced with the descriptors for AFPs with the error limit of <0.05

Architecture	No. of training cycles	Average error	Relative normalized network error for AFPs		Most important molecular descriptors
			Lowest	Highest	
12-4-1	1845	0.00999	Pe-AFP1	WGA	11,3,7
12-5-1	1450	0.01600	Coccinin	Pe-AFP2	7,3,2
12-6-1	690	0.00990	Cicerarin	PAFPs	11,7,3
12-7-1	681	0.00998	Dermaseptin	WGA	7,12,3
12-8-1	790	0.00996	HP	PAFPs	11,7,4
12-9-1	924	0.00940	Pe-AFP1	PAFPs	7,3,6
12-10-1	816	0.00996	Cicadin	WGA	4,7,1
12-11-1	907	0.00910	Cicerarin	Halocidin	3,11,7
12-12-1	650	0.00970	Cicadin	DSP	5,7,11
12-13-1	851	0.00992	Pleurostrin	DSP	6,7,11
12-14-1	682	0.00962	Dermaseptin	WGA	11,3,7
12-15-1	875	0.00993	HP	Halocidin	3,7,11
12-30-1	1918	0.09580	Dermaseptin	WGA	7,3,11
12-50-1	794	0.00998	Cicerarin	DSP	3,11,7

2005). Range of observed activity was between 0.2 to 100. A standard feed-forward network, with back propagation rule and with single hidden layer architecture was chosen. In order to address the problem of possible over-fitting in the training phase, which is usually produced by more weights due to higher numbers of neurons in input and hidden layers, the number of neurons was kept minimum and in addition, we created artificial datasets with exactly same attributes but randomly permuted class labels. This is typically referred to as the Y-randomization test. However, to produce the optimum architecture, powerful enough to model the functions and not create errors more than 0.05%, the number of hidden layer neurons were varied from 1 to 50 (Schneider and Wrede, 1994). Cross validation is done by Leave-some-Out method. The following architectures were produced that met the error limit condition using least number of calculation cycles (Table 2). Higher numbers of hidden layer did not improve the performance. After training ANN, for various architectures the average error, training cycle, relative normalized network error for compound, order of importance of molecular descriptor have been recorded. Results are shown in Table 2.

RESULTS

The results of this study are based on investigation and analysis of collected or calculated data of several antifungal peptides' structural descriptors. Artificial neural network was performed to build a powerful model for prediction of lead and template antifungal peptides. Table 2 shows the results of various architectures of neural network. The number of hidden layer neurons was varied from 1 to 50. Architectures 12-1-1, 12-2-1 and 12-3-1 did not produce any reliable network. Higher numbers of the hidden layers did not improve the performance and decreased the speed of calculation. The best architecture, in terms of cycles of calculation was 12-12-1. The importance value for molecular descriptor is relative to the greatest sum of absolute weights connected to the next layer of the architecture. Therefore, importance of an input descriptor is determined by the sum of the absolute values of the weights of all the outgoing architecture connections from the input node to the next layer. The most important factors were determined as Log P, α-helix content and relative amphipathicity. The least important descriptor was determined as the number generated by placing sum of amino acids between positive amino acids sequentially along each other at pH = 7. Range of predicted activity varied from 2 to 90.32. The correlation coefficients between the experimental and the predicted IC_{50} value pertaining to all the AFPs was 0.89 (Fig. 2).

PeptidesWGA(KPCGKDAGGTVCTNNYCCSKWGS CGIGPGYCGAGCQSGGCDG),PAFPs(AGCIKNGGRCNAS AGPPYCCSSYCFQIAGQSYGVCKNR) and DSP (AVRIGP CDQVCPRIVPERHECCRAHGRSGYAYCSGGGMYCN) corresponded to the highest error that was generated during the training cycles. The least error values were attributed to peptides Cicerarin (VKSTGRADD DLAVKTKYLPP),Pe-AFP1 (QSERFEQQQQDFSHDERFL SQAA) and Cicadin (EYHGFVDKANNENKRKKQQGRDD FVVKPNNFANRRRKDDYNENYYDDVDAADVV). Descriptor charge at pH = 7 had highest relative sensitivity and descriptor length had the least sensitivity. Y-randomization result showed that the classification accuracy for randomized datasets was significantly lower than for the original datasets (data not shown); and hence we concluded that there is no evidence of over-fitting in our models (Cross validation is done by leave-some-out validating method. Validation showed that average of absolute errors was 0.1103. At the end, as positive control, an external set that contained data of 10 antifugal peptides, was simulated by this network. The resulted R^2 factor was 0.84.

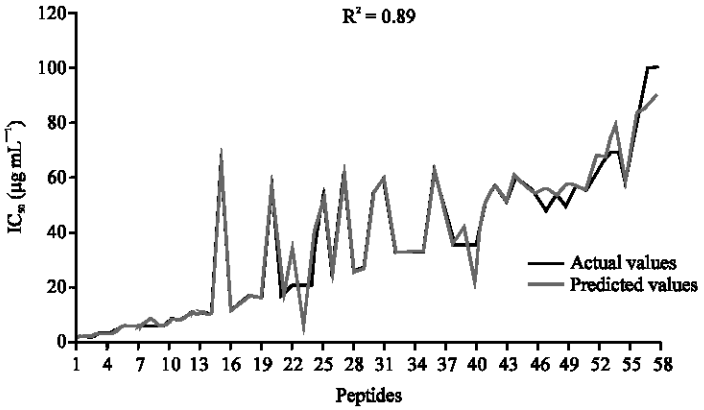

Fig. 2: Plot of predicted values versus observed values for antifungal activity of studied peptides

DISCUSSION

Over 100 AMPs have been found with various activities against pathogenic fungi. These are naturally occurring antifungal peptides. AFPs have captured the attention of researchers because of their application in human health and economic benefits through inhibition of serious damage to crops as a result of fungal infections. AFPs have been identified in many organisms. Attempts have been made to develop methods and strategies for designing effective antimicrobial peptides (Frecer and Ding, 2004). Determination of the most effective structural parameters is very important for scientists in designing *de novo* AFPs. There are one major challenges for developing a suitable method for designing a typical AFP, for example, the antifungal peptides have great amount of variation in size; therefore machine learning applications need fixed length pattern. This problem is similar to B-cell epitope prediction (Saha and Raghava, 2006). Thus we used structural parameters, as these play an important role in antifungal activity. These methods are likely to help the researchers in finding and designing better peptide-based antibiotics. Artificial neural network, which has been recognized as a powerful tool capable of performing better than conventional mathematical models, particularly for the case of nonlinear and multiple processing systems, is one of the widely studied areas within artificial intelligence (Bourquin *et al*., 1998). The results of this study support the ability of ANN algorithm in assigning the biological and physicochemical descriptors to the activity prediction of AFPs. According to the results, in addition to the percent of alpha helical content as the most important parameter in AFP bioactivity, charge positivity and relative amphipathicity, Log P and random coil content could have crucial contribution on IC_{50} of AFPs. The antimicrobial activity of cationic peptides can be modulated through alteration of the peptide hydrophobicity or net charge. Studies have demonstrated that high levels of hydrophobicity can decrease selectivity between the desired bacterial targets and host cells (Kustanovich *et al*., 2002; Zelezetsky *et al*., 2005). Similarly, incorporation of charged residues above a certain maximum (varying with each peptide) does not lead to an increase in activity (Dathe and Wieprecht, 1999). Thus, this balance of charge and hydrophobicity can be delicate and must be empirically determined for each series of peptides. Based on these considerations, it is not surprising that there is a strong correlation between peptide cationicity and antimicrobial activity, since it has been demonstrated in previous studies (Matsuzaki, 1998). However, this relationship is not entirely linear, with examples of direct, indirect, or inverse relationships between these variables (Blondelle and Houghten, 1992). Within a certain range, increasing peptide cationicity is generally associated with increasing antimicrobial potency. Studies with magainin 2 analogs, in which other parameters such as peptide hydrophobicity and helicity were kept constant, have shown that increasing the charge from +3 to +5 results in increasing antibacterial activities against pathogens (Scott and Hancock, 2000). However, there is a limit beyond which increasing positive charge no longer confers increased activity. For the magainins described above, a net charge of +6 to +7 led to an increased hemolytic propensity and a loss of antimicrobial activity (Ganz, 2001). This decrease in antimicrobial activity may result in part from excessively strong peptide interactions with phospholipid head groups, thereby preventing translocation of the peptide into the cell interior. Information about effect of secondary structure of AFPs on biological activity of peptides is incomplete. Many bacterially produced peptides, for instance, have two domains, one of which is α-helical in nature while the other has a β-structure (Uteng *et al*., 2003). For many peptides these secondary

structures are observed only when the peptides interact with membranes; e.g., bovine neutrophil indolicidin is unstructured in an aqueous environment but adopts a boat-like conformation, so that the N and C termini were drawn closer together, when interacting with membranes and membrane mimetics such as sodium dodecyl sulfate and dodecyl phosphocholine (Rozek *et al*., 2000).The plasticity of the secondary structure of indolicidin has been suggested to permit different interactions with different molecules, including DNA and membranes (Hsu *et al*., 2005). This indolicidin shape could also be stabilized by adding a cysteine residue to each end and creating a disulfide bridge (Rozek *et al*., 2003). Consequently, the inclusion of a particular peptide into a structural group does not give an indication as to its mode of action or its spectrum of activity. In fact, some peptides with very similar secondary structures have had quite opposite characteristics (Hancock and Patrzykat, 2002). Thus, like for antibacterial peptides, there are no obvious conserved structural domains that give rise to antifungal activity. It should be mentioned here that there are a few studies that emphasize on the role of random coil and beta turn in AMPs' bioactivity and these topics must be investigated in future research. The plasticity of the secondary structure of indolicidin has been suggested to permit different interactions with different molecules, including DNA and membranes. Beta turn and randomcoil have a great effect on protein plasticity (Hsu *et al*., 2005). This feature, together with other structures plays an important role in the orientation of antimicrobial peptides toward lipid bilayer membrane (Besson and Michell, 1987). For example, by changing the membrane-associated shape of indolicidin so that the N and C termini were drawn closer together, (more extended strand structure formed) the activity against gram-negative bacteria was increased (Rozek *et al*., 2003).

Previous results showed that residue 1 (almost glycine) and 17 (occupied almost by small amino acids like valine, argenine and glycine) have the most important role among the other residues on the AFPs bio-activity, (Sardari *et al*., 2007, unpublished data). Previously Sneh *et al*. (2007) showed that for antimicrobial peptides certain residues are more abundant at specific positions, e.g., G, F, V, R at the first position; L, I, W, F at the second position and similarly, certain residues are preferred at the N-terminus, for example residues K, G, C and R are preferred at most of the positions. About the role of various regions of AFPs it should be mentioned that Lys-54/55 and Lys-71/72 on the putative helices are critical for antimicrobial activity and the C-terminal last 3 amino acids are important for the structural integrity of the C-terminal region and for effective antimicrobial activity. The C-terminus interacts with the cell membrane and makes a pore, whereas the N-terminus helps in bacterial specific interaction process. In the same way, length of peptide plays a vital role in AFP activity. The observations suggest that peptide-mediated wall disruption may occur as a result of several independent or cooperative mechanisms of action. Furthermore individual factors such as ion concentrations, the presence or absence of other immune mechanisms or synergistic exogenous antimicrobial agents may intervene. In spite of the above-mentioned facts, knowing the main mechanism that play the major role would be of great value. The parameters that assessed as the main factors in AFP bioactivity of this study, are the main elements in BSPM as well. As mentioned before, BSPM relied on distinct alpha helical structure for the peptide and effective hydrophobic interaction of the peptide with target membrane. So, these results introduce BSPM as the main mechanism of AFP action. These findings would be helpful in design of more efficient AFPs, a field of research that is in the center of many attempts for providing novel antifungal agents.

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