International Journal of Academic Research and Development
ISSN: 2455-4197
Impact Factor: RJIF 5.22
www.academicsjournal.com
Volume 2; Issue 6; November 2017; Page No. 165-170

Antifungal activity of human neutrophil peptides (HNP-1, HNP-2 and HNP-3) against glucuronoxylomannan (GXM) of *Cryptococcus neoformans*: An *In-silico* study

Gunjan Uttam, Neelabh, *Karuna Singh
Department of Zoology (MMV), Banaras Hindu University, Varanasi, Uttar Pradesh, India

Abstract
Emergence of resistance against existing antifungal drugs created a need to find out new structural classes of antifungal agents. Defensins are significant vertebrate antifungal peptides which target the capsule of *Cryptococcus neoformans*. The cryptococcal capsule is an important virulence factor and its main component is the polysaccharide glucuronoxylomannan (GXM). In this study, we design a new structural class of antifungal peptidomimetics of human neutrophil peptides (defensins). Bioinformatics tools were used to predict level of sequence similarity amongst them. Peptidomimetics of human neutrophil peptides (HNPs) were designed which showed better binding with GXM of *Cryptococcus* than natural human neutrophil peptides (HNPs) and could be used as potential drug candidates against cryptococcal infections.

Keywords: peptidomimetics, antifungal peptides, glucuronoxylomannan, bioinformatics, human neutrophil peptides

1. Introduction
The incidence of invasive fungal infections such as candidiasis, cryptococcosis, and aspergillosis in humans is increasing rapidly [1]. Fungal infections are causes of mortality and morbidity, especially in patients whose immune systems are compromised by AIDS, cancer, or organ transplant [2]. *Cryptococcus neoformans* commonly causes cryptococcosis and cryptococcal meningitis. Center for Disease Control, USA, evaluated the occurrence of approximately one million new cases of cryptococcal meningitis each year resulting in the death of 625,000 people worldwide [3, 4]. Usually the infection of *Cryptococcus* starts with lungs but patients having advanced immune suppression end up having cryptococcal meningitis [4].
The common treatment for cryptococcosis in patients suffering from asymptomatic or mild to moderate pulmonary infections is fluconazole. Amphotericin B in combination with flucytosine is prescribed for treatment scheme of severe lung infections which is slightly different from the treatment with fluconazole [5]. But in the recent past, some isolates of *Cryptococcus* have shown resistance against these drugs and patients have witnessed relapse or failure on treatment with flucytosine, fluconazole and amphotericin B [6]. Moreover, the treatments have their respective drawbacks, such as drug related toxicity, emergence of resistant strains, nonoptimal pharmacokinetics, poor solubility, and serious drug−drug interactions [7]. Therefore, there is an indispensable need to discover or design new antifungal agents that do not share the same structural platform and the same line of mechanism as existing drugs [8]. It is saddening to state that most prevalent pathogens like *Cryptococcus* are developing resistance against the newly discovered antifungal drugs [9].
Peptides having a broad spectrum antifungal activity are one of the solutions to the above problem [10]. Antifungal peptides (AFPs) are one of the examples of antimicrobial peptides which are integral components of the innate immune system of eukaryotes and are responsible for inhibiting the growth of the fungi [10, 11]. These are defined as the peptides that are accountable for inhibiting the growth of fungus [12]. Defensins, lactoferrins, histatins, cathelicidins and tritrpticins are AFPs obtained through various components of the mammalian body and possess different structures and slightly different modes of action [11, 13].
Defensins are significant vertebrate antimicrobial peptides that are reported to inhibit the fungi [13]. They are cationic in nature with small-molecular-mass (3 to 4 kDa). Defensins are found in the azurophilic granules of neutrophils in rabbits, guinea pigs, rats, and humans [14-18]. Structurally, they consist of 29 to 34 amino acids with six conserved cysteine residues, accounting for three disulfide bonds [15, 17, 19, 20]. Defensins are thought to act by permeabilizing the cell membrane of microorganisms. Cationic nature of the peptides makes them adhere to the cell membranes of the microorganisms by forming multimeric pores that are responsible for leakage of materials of the cell [21-24]. Some of the important human defensins along with their targets and the number of amino acids in their sequences are summarized in table 1 [15, 25-27].

Table 1: Types of defensins and their salient features

Defensins	Number of amino acids	Target organisms	In vitro MIC (lg/ml)
HNP-1	30	*C. albicans*	50
HNP-2	29	*C. albicans*	50
HNP-3	30	*C. neoformans*	50.0 (LD50b)

Many factors such as capsule, melanin formation, phospholipase, mannitol, urease and proteinases are responsible for virulence or pathogenicity of *Cryptococcus neoformans* [28]. Cryptococcal capsule has been recognized as a crucial virulence factor which is made up of the

polysaccharide glucuronoxylomannan (GXM) [29]. GXM has an α-1,3-linked mannose backbone which is O acetylated and substituted with single side chains of xylose and glucuronic acid [30,31]. There are several direct and indirect lines of evidence for binding and/or uptake of GXM by neutrophils [32]. GXM blocks binding of CD18 antibodies to human neutrophils, suggesting an interaction between GXM and neutrophil CD18 [33].

The aim of this study is to design antifungal peptidomimetics of human neutrophil peptide (HNP-1, HNP-2 and HNP-3) and their interaction with glucuronoxylomannan of cryptococcal capsule using bioinformatics approaches.

2. Materials and Methods

Identification of conserved regions in amino acid sequence of human neutrophil peptides

Amino acid sequences of human neutrophil peptide (HNP-1, HNP-2 and HNP-3) were retrieved from NCBI (National Center of Biotechnology Information, www.ncbi.nlm.nih.gov) in FASTA format. ClustalX (http://www.clustal.org) software was used for identification of conserved regions. The evolutionary relationship between the different AFPswas studied by the Molecular Evolutionary Genetic Analysis version (MEGA 6) (www.megasoftware.net) software.

Interaction between structures of human neutrophil peptides (HNPs) and glucuronoxylomannan (GXM)

The protein structures of natural human neutrophil peptide (HNP-1, HNP-2 and HNP-3) were downloaded from Protein Data Bank (PDB) (https://www.rcsb.org). Protein structure of glucuronoxylomannan (GXM) of *Cryptococcus* was downloaded from NCBI and then designed by Phyre2 web server (www.sbg.bio.ic.ac.uk/~phyre2/). RAMPAGE server (mordred.bioc.cam.ac.uk/~rapper/rampage.php) was used for Ramachandran Plot Analysis for the predicted GXM structure. Docking software PatchDock (https://bioinfo3d.cs.tau.ac.il/PatchDock) and FireDock (bioinfo3d.cs.tau.ac.il/FireDock) were used to determine the interactions between structures of human neutrophil peptides (HNP-1, HNP-2 and HNP-3) and designed GXM. Hydrogen bond and binding energies of human neutrophil peptides and GXM were observed through the UCSF Chimera package (https://www.cgl.ucsf.edu).

In-silico designing of peptidomimetics of Human Neutrophil Peptides (HNP-1, HNP-2 and HNP-3)

Peptidomimetics were designed by altering the structures of human neutrophil peptides (HNP-1, HNP-2 and HNP-3) using ChemSketch (https://chemsketch.en.softonic.com) and Open Babel (http://openbabel.org) tools. ChemSketch is a molecular modeling program used to create and modify images of chemical structures. Open Babel is used to interconvert chemical file formats. Furthermore, isosteres were used to modify these structures.

Interaction between mimetics of human neutrophil peptides and glucuronoxylomannan (GXM)

With the help of PatchDock and FireDock, binding energies were determined between mimetics of human neutrophil peptides and GXM. Comparison between binding energies of modified HNPs and natural HNPs elucidate the effectiveness of the altered HNPs over the natural ones.

3. Results & Discussion

Identification of conserved regions in amino acid sequence of human neutrophil peptides

Amino acid sequences of human neutrophil peptides **(**HNP-1, HNP-2 and HNP-3) were retrieved from NCBI in FASTA format (Table 2). The protein sequences were aligned using ClustalX. ClustalX is a windows interface for the multiple sequence alignment programs. Through it, we observed that observing about 98% similarity was found between HNPs (Fig. 1). Phylogenetic tree of human neutrophil peptides **(**HNP-1, HNP-2 and HNP-3) shows the evolutionary pattern as shown in Fig. 2. Neelabh *et al.* (2016) [10] used T-Coffee (Bioinformatics tool) to achieve evolutionary/sequential organization. The results produced by them shows that defensins from various organisms (mammals) were only 15 % dissimilar in their sequential organization.

Table 2: List of retrieved amino acid sequence of human neutrophil peptides (HNP-1, HNP-2 and HNP-3) from NCBI

Defensins	No. of amino acids	Sequence
HNP-1	30	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-2	29	CYCRIPACIAGERRYXTCIYQGRLWAFCC
HNP-3	29	DCYCRIPACIAGERRYGTCIYQGRLWAFCC

Fig 1: Multiple sequence alignment results of various defensins (HNPs) using the multiple sequence alignment software ClustalX

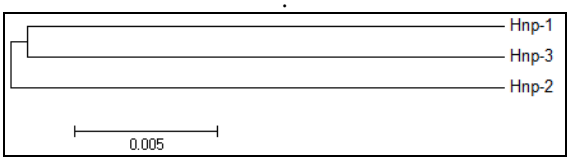

Fig 2: Phylogenetic tree of human neutrophil peptides **(**HNP-1, HNP-2 and HNP-3) using MEGA software.

Interaction between structures of human neutrophil peptides (HNPs) and glucuronoxylomannan (GXM)

PDB structures of human neutrophil peptides **(**HNP-1, HNP-2 and HNP-3) have been downloaded from Protein Data Bank (PDB) as shown in Fig. 3. Structure of GXM was modeled through Phyre[2]. Phyre[2] is an automatic fold recognition server for predicting the structure and/or function a protein sequence. The structure obtained was validated through RAMPAGE (Fig 4). Phyre[2] webserver has been used in number of studies like modeling of beta tubulin of *C. neoformans* [34]. Binding energies between natural human neutrophil peptides **(**HNP-1, HNP-2 and HNP-3) and GXM have been shown in table 3. PatchDock & FireDock webserver have been used in many studies for determining the binding energies between different proteins and ligands. One such study involves the determination of binding energy between Acyclovir and thymidine kinase enzyme of Herpes Simplex Virus [35].

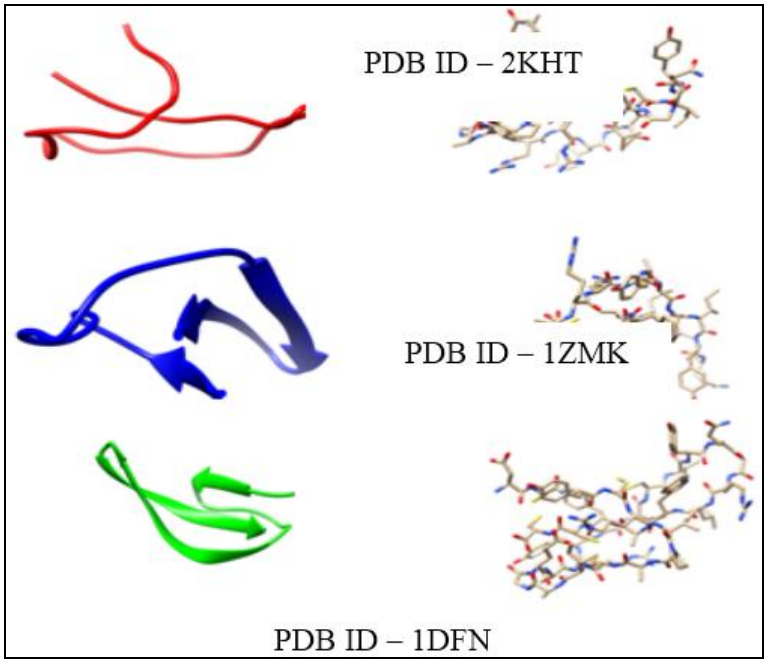

Fig 3: PDB structures (Ribbon and Atom bond) of human neutrophil peptides **(**HNP-1, HNP-2 and HNP-3 are shown in red, blue and green respectively).

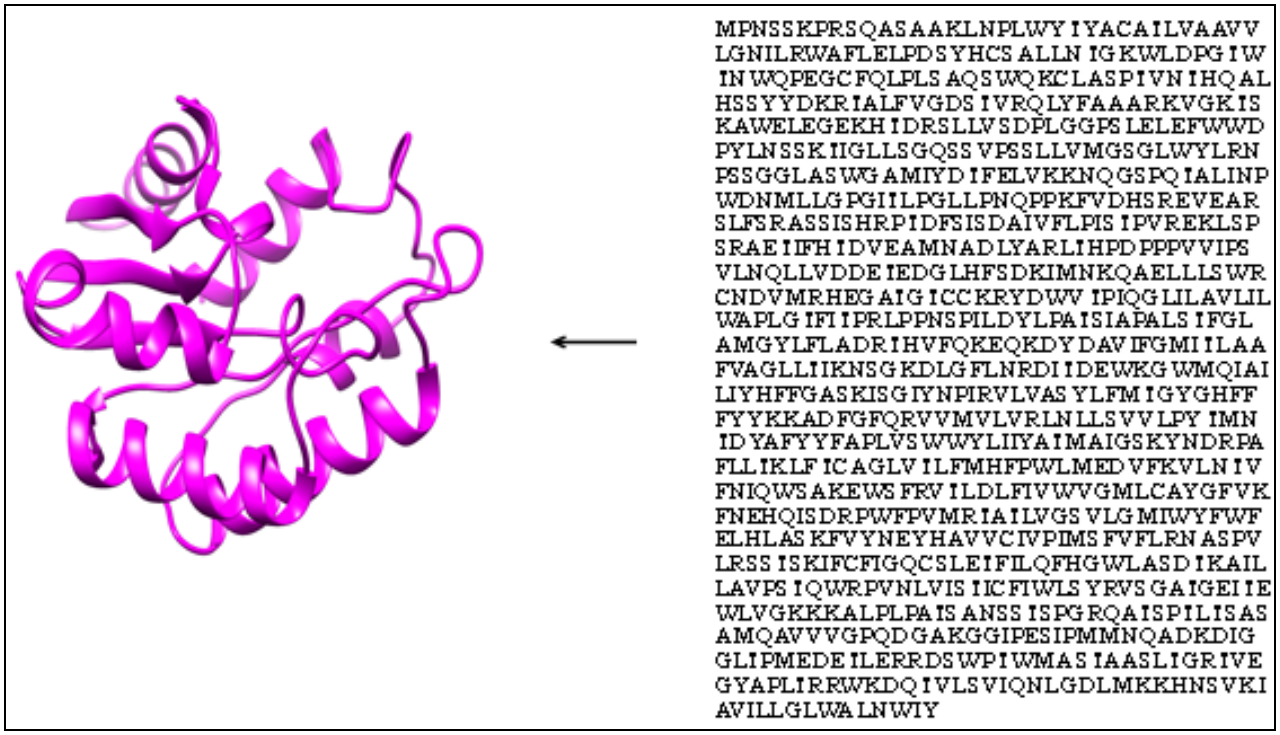

Fig 4: Modeled structures and sequence of Cryptococcal GXM (O-acetyltransferase CAS1) using Phyre[2] webserver

Table 3: Binding energies between PDB structures of human neutrophil peptides and GXM.

Interaction	Lowest Binding Energy(kcal/mol)	Hydrogen bond
HNP-1 and GXM	-30.99	1
HNP-2 and GXM	-33.27	0
HNP-3 and GXM	-17.39	0

***In-silico* designing of the peptidomimetics of Human Neutrophil Peptide (HNP- 1, HNP-2 and HNP-3)**

Peptidomimetics of Human Neutrophil Peptide (HNP-1, HNP-2 and HNP-3) were designed using bioinformatics approaches with the help of isosteres. Isosteres are molecules or ions of similar size containing the same number of atoms and valence electrons, used to produce compounds that can sometimes have similar biological activities. Designed peptidomimetics were shown below in fig. 5. And changes were encircled by colored circles. Red color circles indicate a replacement of OH by SH, green color indicates that H is replaced by F and pink color circle indicates that –NH2- is replaced by –CH2-. In 2015, Mahindra *et al.* reported the synthesis of a new structural class of short antifungal peptidomimetics having an activity better than the control drug Amphotericon B against *C. neoformans* [36].

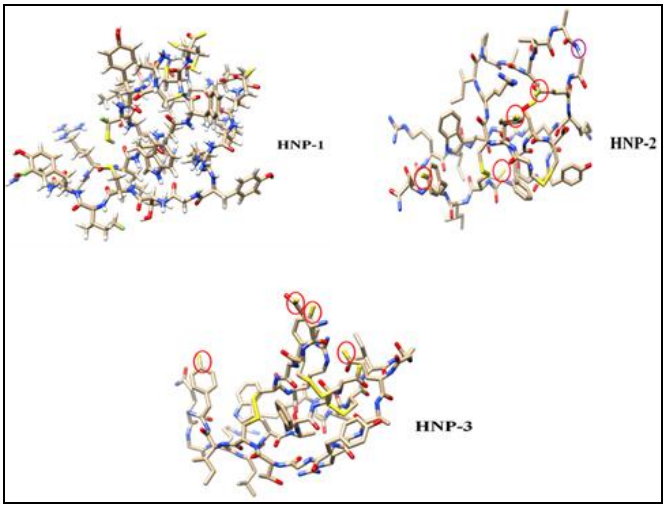

Fig 5: Designed mimetics of human neutrophil peptides

Interaction between altered structures (peptidomimetics) of human neutrophil peptides and glucuronoxylomannan (GXM)

Interaction between peptidomimetics of human neutrophil peptides and glucuronoxylomannan (GXM) were seen by docking. Binding energies of peptidomimetics against GXM were found to be better than binding energies of natural human neutrophil peptides against GXM (Table 4). Number of hydrogen bond between peptidomimetics of human neutrophil peptides and glucuronoxylomannan (GXM) has been shown in fig. 6.

Table 4: Binding energies between altered structures of HNPs and GXM.

Altered structures	Lowest Binding Energy(kcal/mol)	Hydrogen bond
Altered HNP-1	-58.78	1
Altered HNP-2	-36.65	2
Altered HNP-3	-24.99	3

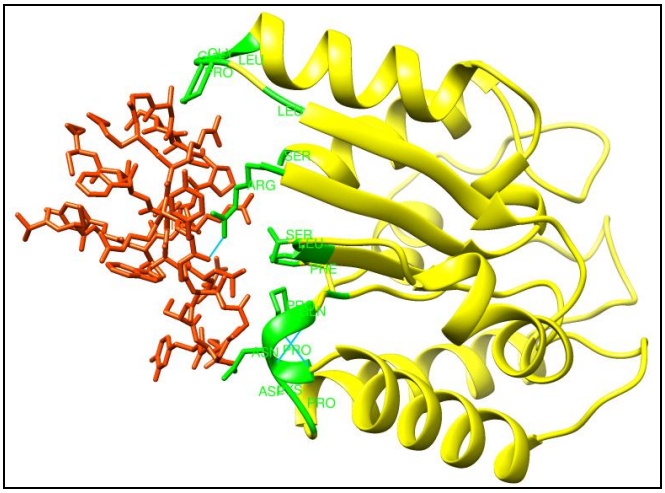

Fig 6: (a) Interaction of modified structure of HNP-1 with GXM (yellow in color), (hydrogen bonds - blue lines).

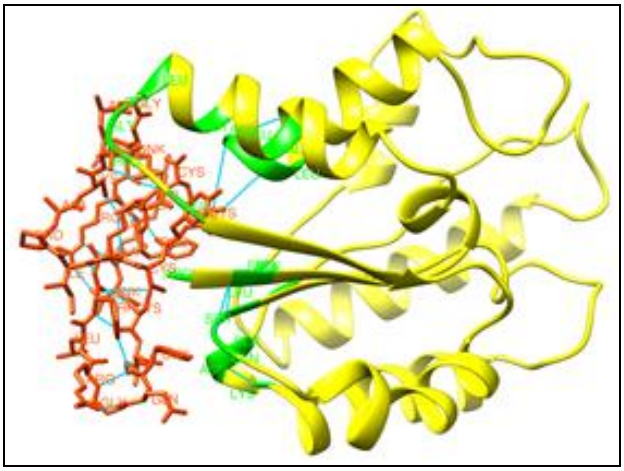

Fig 6: (b) Interaction of modified structure of HNP-2 with GXM (yellow in color), (hydrogen bonds - blue lines).

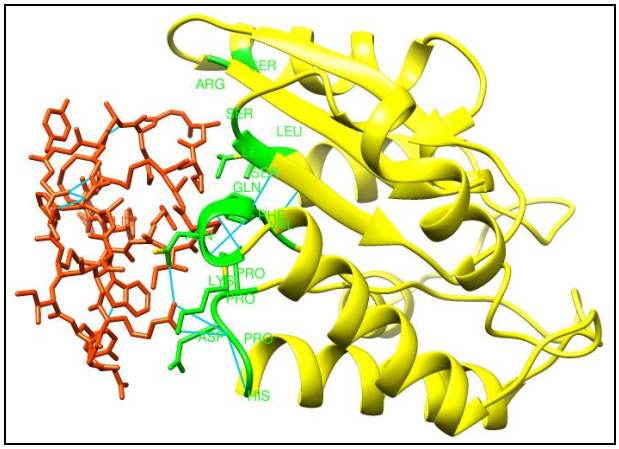

Fig 6: (c) Interaction of modified structure of HNP-3 with GXM (yellow in color), (hydrogen bonds - blue lines).

4. Conclusion

Multiple sequence alignment and designing of peptidomimetics of antifungal peptides (human neutrophil peptides) were performed using *In-silico* approach. The mimetics of human neutrophil peptides (HNP-1, HNP-2 and HNP-3) were designed which bind more efficiently with GXM than human neutrophil peptides. However, its wet lab validation is required. Moreover, this study facilitates the generation of novel anti-fungal drugs/vaccines that could prevent cryptococcal infections in their early stage and would certainly be beneficial for mankind.

5. Acknowledgments

The authors are thankful to the Council of Science and Technology, U.P. for funding this study under the project code no. P-26/121. The authors are also thankful to The Principal, MMV, BHU for providing infrastructure facilities.

6. References

1. Mahindra A, Bagra N, Wangoo N, Khan SI, Jacob MR, Jain R. Discovery of short peptides exhibiting high potency against Cryptococcus neoformans, *ACS medicinal chemistry letters*, 2014; 5(4):315-320.
2. Groll AH, Lumb J. New developments in invasive fungal disease, Future Microbiol. 2012; 7:179-184.
3. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS, AIDS 2009; 23:525-530. doi: 10.1097/QAD.0b013e328322ffac
4. CDC C. neoformans Infection Statistics|Fungal Diseases, 2015. Available at: http://www.cdc.gov/fungal/diseases/cryptococcosis-neoformans/statistics.{\p enalty-\@M}html[AccessedFebruary4, 2016].
5. CDC C. neoformans Infection Statistics|Fungal Diseases, 2014. Available at: http://www.cdc.gov/fungal/diseases/cryptococcosis-neoformans/statistics.{\p enalty-\@M}html[AccessedFebruary4, 2016].
6. López-Jodra O, Torres-Rodríguez JM, Mendez-Vasquez R, Ribas-Forcadell E, Morera-López Y, Baro-Tomas T, Alia-Aponte C. In vitro susceptibility of *Cryptococcus neoformans* isolates to five antifungal drugs using a colorimetric system and the reference microbroth method, Journal of Antimicrobial Chemotherapy. 2000; 45(5):645-649.
7. Georgopapadakou NH. Antifungals: Mechanism of action and resistance, established and novel drugs, Curr. Opin. Microbiol. 1998; 1:547-557.
8. Arnusch CJ, Albada HB, Van Vaardegem M, Liskamp RMJ, Sahl HG, Shadkchan Y, Osherov N, *et al*. Trivalent ultrashort lipopeptides are potent pH dependent antifungal agents, J Med. Chem. 2012; 55:1296-1302.
9. Avrahami D, Shai Y. A new group of antifungal and antibacterial lipopeptides derived from non-membrane active peptides conjugated to palmitic acid, Journal of Biological Chemistry. 2004; 279(13):12277-12285.
10. Neelabh Singh K, Rani J. Sequential and Structural Aspects of Antifungal Peptides from Animals, Bacteria and Fungi Based on Bioinformatics Tools. Probiotics and antimicrobial proteins, 2016; 8(2):85-101.
11. van der Weerden NL, Bleackley MR, Anderson MA. Properties and mechanisms of action of naturally occurring antifungal peptides, Cell Mol Life Sci. 2013; 70:3545-3570.
12. Gallo RL., Victor N. Endogenous production of antimicrobial peptides in innate immunity and human disease, Curr Allergy Asthma Rep. 2003; 3:402-409.
13. De Lucca AJ, Walsh TJ. Antifungal peptides: novel therapeutic compounds against emerging pathogens, Antimicrob Agents Chemother. 1999; 43:1-11
14. Eisenhauer P, Harwig S, Szklarek D, Ganz T, Selsted M, Lehrer R. Purification and antimicrobial properties of three defensins from rat neutrophils, Infect. Immun. 1989; 57:20212027.
15. Ganz T, Selsted M, Lehrer R. Defensins, Eur. J Haematol, 1990; 44:1-8.
16. Selsted M, Harwig S. Purification, primary structure, and antimicrobial activities of a guinea pig neutrophil defensin, Infect. Immun. 1987; 55:2281-2286.
17. Selsted M, Harwig S, Ganz T, Schilling J, Lehrer R. Primary structures of three human neutrophil defensins, J Clin. Invest. 1985; 76:1436-1439.

18. Selsted M, Szklarek D, Lehrer R. Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes. Infect. Immun, 1984; 45:150-154.
19. Lehrer R, Ganz T, Selsted M. Defensins: natural peptide antibiotics from neutrophils, ASM News. 1990; 56:315-318.
20. Selsted M, Harwig S. Determination of the disulfide array in the human defensin HNP-2, a covalently cyclized peptide, J Biol. Chem. 1989; 264:4003-4007.
21. Lehrer RI, Barton A, Daher KA. *et al.* Interaction of human defensins with Escherichia coli. Mechanism of bactericidal activity J Clin Invest. 1989, 84:553.
22. Lehrer RI, Szklarek D, Ganz T, *et al.* Correlation of binding of rabbit granulocyte peptides to Candida albicans with candidacidal activity, Infect Immun. 1985, 49:207-211.
23. Patterson-Delafield J, Szklarek D, Martinez RJ, *et al.* Microbicidal cationic proteins of rabbit alveolar macrophages: amino acid composition and functional attributes, Infect Immun. 1981; 31:723-731.
24. White SH, Wimley WC, Selsted ME. Structure, function, and membrane integration of defensins. Curr Opin Struct Biol. 1995; 5:521-527.
25. Joly S, Maze C, McCray PB. Human b-defensins 2 and 3 demonstrate strain-selective activity against oral microorganisms, J Clin Microbiol. 2004; 42:1024-1029.
26. Schro ¨der JM, Harder J. Human beta-defensin-2, Int J Biochem Cell Biol, 1999; 31:645-651.
27. Hoover DM, Wu Z, Tucker K, *et al.* Antimicrobial characterization of human b-defensin 3 derivatives, Antimicrob Agents Chemother. 2003; 47:2804-2809.
28. Buchanan KL, Murphy JW. What makes *Cryptococcus neoformans* a pathogen?, Emerg. Infect, 1998; 4:71-83. doi:10.3201/eid0401.980109.
29. Kozel TR, Mastroianni RP. Inhibition of phagocytosis by cryptococcal polysaccharide: dissociation of the attachment and ingestion phases of phagocytosis, Infect. Immun. 1976; 14:62
30. Bhattacharjee AK, Bennett JE, Glaudemans CPJ. Capsular polysaccharides of Cryptococcus neoformans. Rev. Infect. Dis. 1984; 6:619-624.
31. Cherniak R, Valafar H, Morris LC, Valafar F. *Cryptococcus neoformans* chemotyping by quantitative analysis of 1H nuclear magnetic resonance spectra of glucuronoxylomannans with a computer-simulated artificial neural network. Clin. Diagn. Lab. Immunol. 1998; 5:146-159.
32. Chang ZL, Netski D, Thorkildson P, Kozel T. Binding and internalization of glucuronoxylomannan, the major capsular polysaccharide of Cryptococcus neoformans, by murine peritoneal macrophages, Infection and immunity. 2006; 74(1):144-151.
33. Dong ZM, Murphy JW. Cryptococcal polysaccharides bind to CD18 on human neutrophils, Infect. Immun. 1997; 65:557-563.
34. Neelabh, Karuna S. In silico studies on the effect of griseofulvin on tubulin protein of Cryptococcus neoformans and its in vitro validation. Journal of Microbiology, Biotechnology and Food Sciences. 2017; 6(6):1280-1283.
35. Neelabh, Jeswara K, Kumari A, Singh K. *In-silico* designing of NKK: A better ligand than Aciclovir against Herpes Simplex Virus. Indian Journal of Pharmaceutical and Biological Research, 2015; 3(1):48.
36. Mahindra A, Bagra N, Wangoo N, Khan SI, Jacob MR, Jain R. Discovery of short peptides exhibiting high potency against Cryptococcus neoformans. ACS medicinal chemistry letters. 2014; 5(4):315-320.