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Original Article

Cyclopeptide Kalata B12 as HCV-NS5A potent Inhibitor

Faiza Shams[1], Nazia Kanwal[2], Somayya Tariq[1], Ayesha Malik[1], Kausar Malik[1] and Bushra Ijaz[1]*

[1]Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan
[2]Superior University, Lahore, Pakistan

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***Corresponding Author:**
Bushra Ijaz
Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan
bijaz@cemb.edu.pk

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ABSTRACT

Hepatitis C Virus (HCV) is the leading cause of liver diseases globally, causing severe complications such as liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Despite the advent of successful regimens, still, 71 million individuals are chronically infected every year. Therefore, more accessible novel therapies are needed to fight the challenges such as adverse effects, genotype selectivity, and resistance to these regimens due to viral mutations. HCV NS5A is a non-structural phosphoprotein, with its pivotal role in viral replication assembly, and has been the target of continuous research. Cyclopeptides are an emerging class of peptides reported to have antiviral, anticancer, and antimicrobial properties. These cyclopeptides have exceptional resistance to thermal, chemical, or enzymatic degradation. Herein, we present the inhibitory potential of cyclopeptide Kalata B12 against the HCV NS5A gene. **Objective:** To investigate the antiviral potential of Kalata B2, Kalata B12, and cycloviolacin O14 against HCV NS5A **Methods**: We investigated thirty cyclopeptides through molecular docking analysis for their anti-HCV-NS5A inhibition potential. Three cyclopeptides, Kalata B2, Kalata B12, and cycloviolacin O14 showed minimum binding energies, for their antiviral potential. The defense-related, circular mini-protein Kalata B12 showed an impressive docking score of -9.80 Kcal/mol. Further, it was synthesized and went through cytotoxicity analysis via MTT assay on HepG2 cell line, which showed more than 85% cell viability at submicromolar concentrations. **Results:** The peptide Kalata B12 showed significant *(***P<0.0001)* inhibition of NS5A gene (approx. 75%) at 100nM in *In vitro* trials, confirmed by real-Time PCR analysis. **Conclusions:** Kalata B12 cyclopeptide was found to be a potential HCV NS5A inhibitor.

INTRODUCTION

Hepatitis C Virus (HCV) is the leading cause of acute and chronic liver diseases imposing a serious public health burden worldwide. It is estimated that approximately 200 million people (3% of the world population) are chronically infected with this virus [1,2]. HCV is a positive, single-stranded RNA virus belonging to the *Flaviviridae* family that infects the liver cells causing liver fibrosis, cirrhosis, and Hepatocellular carcinoma (HCC) [3]. Previously standard therapy for HCV treatment was a combination of pegylated interferon (INFα) and Ribavirin (RBV) with less than 50% sustained virological response rate (SVR) [1]. With the discovery of direct-acting Antivirals (DAAs), higher SVR has been achieved with more than 90% cure rate in a shorter duration of time and fewer adverse effects [4]. Although with the availability of DAAs that directly target the HCV proteins NS3/4A protease, NS5B polymerase, and NS5A, treatment success has been accomplished but there are still some challenges such as high cost, adverse effects, and possible long-term drug resistance to these antivirals due to viral mutations and genotypes variability. So, there is a need to develop more efficient drugs and regimens to overcome these issues. Over the past decades, natural medicinal plant products and compounds have gained attention as therapeutic options against many micro-organisms and diseases[5-7]. Thus, natural plant products and compounds could be the best alternative therapeutic option that can be used alone or with other antivirals to defeat this virus [8]. NS5A is HCV non-structural membrane-associated phosphoprotein [9]. It consists of about 447 to 466 amino acids containing three structural

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domains I, II, and III has 58 kDa molecular weight. It has two phosphorylated forms named as P56 and P58, which plays important role in viral replication and assembly [10,11]. By targeting NS5A protein HCV infection can be inhibited, thus NS5A is a promising target for antiviral drugs. Cyclotides are a unique class of plant-derived peptides emerging as a promising therapeutic approach containing many biological and pharmacological properties. These cyclotides are macrocyclic peptides containing approximately 28-37 amino acids and three disulfides (-S-S-) bonds [12]. They contain a unique cyclic cysteine knot (CCK) motif in which three disulfide bonds and 6 conserved cysteine residues are arranged in a knot forming a cyclic backbone that's why named cyclotides or cyclopeptides. This CCK motif gives them exceptional resistance to high temperature, enzymatic and chemical degradation, and more stability as compared to linear peptides. In plants, they are considered to be part of their natural defense system against pests and insects *etc.*, [13]. Previous scientific studies proved that these circular peptides have many biological activities including cytotoxic, anti-microbial, anti-cancer, anti-bacterial, uterotonic, hemolytic, anti-HIV, *etc. [12]*. In the present study, thirty cyclopeptides from the literature were selected and *in-silico* studies were conducted on them. Based on their energy score of interaction with HCV NS5A genotype 1a, three peptides were selected for *in vitro* studies. Our results show that these cyclotides can be a potent therapeutic option against HCV as they inhibit the HCV NS5A gene. Further investigations are needed to explore the function and activity of these peptides.

METHODS

***In-silico* screening and synthesis of cyclopeptides:** Thirty cyclopeptides with vast pharmacological properties were selected from the literature. *In-silico* studies were carried out via molecular docking software on these cyclopeptides to check affinity with HCV NS5A genotype 3a protein [14]. Three cyclopeptides were selected based on energy score and interaction with the site for further studies. These peptides were synthesized from Karebay Biochem *Inc*. USA in lyophilized form. Then, these peptides were reconstituted in dimethyl sulfoxide (DMSO), (Sigma Aldrich).

***In-vitro* cytotoxicity Analysis of Cyclopeptides:** MTT 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide Assay was performed to measure cell viability in 96 well plates. HepG2 cells were seeded in a Flat bottom 96 well plate at $1x10^4$ density in DMEM medium (Gibco). Then cells were incubated in a humidified environment at 37°C and 5% CO_2(Thermo Fisher Scientific). After 24 hours, upon cell confluency of 80-85% cells were treated with cyclotides at sub-micromolar concentrations (0.1nM to 1µM). DMSO was used as a control. After incubation for 24 hours, cells were washed with 1X PBS (Phosphate Buffer Saline). 10µl MTT dissolved in PBS at 0.5mg/ml concentration was added to each well and incubated for 2-3 hours. Then MTT buffer is removed and formazon is dissolved in DMSO. After 30 min incubation at room temperature absorbance was measured at 540nm and 650nm.

HCV NS5A transfection in HepG2 cells: HepG2 cells were grown in a 24-well plate at a seeding density of $5x10^4$ per well. The next day cells were transfected with HCV NS5a genotype 1a along with cyclopeptides at different concentrations using lipofectamine (Invitrogen™). After 24 hours of incubation at 37°C, total RNA was extracted using Triazole reagent (Invitrogen™ Life Technologies) and subjected to cDNA synthesis using a First-strand cDNA synthesis kit (Invitrogen, US).

NS5 A expression studies: Prepared cDNA was used for NS5A expression studies using semi-quantitative and quantitative Real-Time PCR. Each sample was prepared in triplicate in ABI 7500 Real-Time PCR system (Applied Biosystems, USA). The relative expression level of NS5A was determined using GAPDH as internal control and the fold change was evaluated using the RT-PCR comparative CT method (ΔΔ CT) as previously described [15]. Primer sequences of NS5A and GAPDH are listed in Table 1. Results were analyzed using Graphpad Prism software.

Gene	Primer Sequence
NS5a Forward primer	GGACGACGATGACAAGGACT
NS5a Reverse primer	TTATAGTTCGGCGCAGGAAG
GAPDH Forward primer	CGGATTTGGTCGTATTGG
GAPDH Reverse primer	AGATGGTGATGGGATTTC

Table 1: Sequence of Primers

RESULTS

***In-silico* study of cyclotides:**

In silico study was conducted on the thirty selected peptides using MOE docking software. The docking results revealed three cyclotides best fit in the HCV NS5A proteins pocket. Kalata B2 exhibited the highest binding affinity towards HCV NS5A i.e., -11Kcal/mol while binding GLy896 residue. Further, Kalata B12 and Cycloviolacin O14 also produced promising results against NS5A. Kalata B12 showed the second-lowest binding energy of -9.8Kcal/mol while Cycloviolacin O14 represented a -8.4 Kcal/mol docking score. The former trusses with Thr95 and Thr64 and the latter bind to Thr55, Thr94, and Thr64. The structure and interaction of cyclotides with the NS5A

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binding site are shown in Table 2.

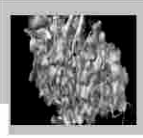
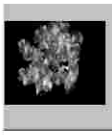
Structure	Interaction of peptide with NS5A site	Binding Energy Score	Reside Involved
Kalata B2		-11 Kcal/mol	Gly898
		-9.80 Kcal/mol	Thr95 Thr64
		-8.4 Kcal/mol	Thr55 Thr94 Thr64

Table 2: Structure and interaction of cyclotides with NS5A site

Properties of cyclopeptides:

Three selected cyclotides were synthesized based on their interaction with HCV NS5A gene. Table 3 shows peptide source, length, nature and their sequences.

Peptide	Source	Length	Nature	Sequence
Kalata B2	*Oldenlandia affinis*	28	Acidic	GLPVCGETCFGGTCNTPGCSCTWPICTRD
Kalata B12	*Oldenlandia affinis*	29	Acidic	GSLCGDTCFVLGCNDSSCSCNYPICVKD
Cycloviolacin O14	*Viola odorata*	31	Basic	GSIPACGESCFKGKCYTPGCSCSKYPLCAKN

Table 3: Properties and sequences of cyclotides

In-Vitro Cytotoxicity Analysis of Cyclotides

To check cytotoxic potential of these selected peptides, HepG2 cells were treated with different concentrations of cyclotides for 24 hours. MTT Assay results showed that all three cyclotides Kalata B2, Kalata B12, and Cycloviolacin O14 were non-toxic as all peptides showed more than 85% cell viability at concentrations from 0.05nM to 1µM.

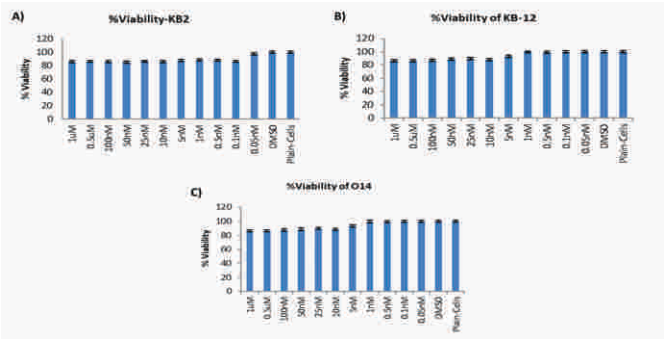


Figure 1: Cytotoxic Analysis of cyclopeptides

HepG2 cells were treated with cyclopeptides for 24 hours. After incubation, MTT assay was performed and measure absorbance at 540 and 650nm.

HCV NS5A expression in response to cyclotides:

HepG2 cells were grown in a 24-well plate. Upon 80-85% confluence, cells were transfected with HCV NS5A and incubated with peptides at different concentrations. After 24 hours, total RNA was extracted and cDNA was synthesized. This cDNA was used for inhibition studies using NS5A gene-specific primers. GAPDH is used as an internal control. All these cyclotides showed considerable inhibition of the NS5A gene. However, Kalata B12 showed more promising results. It showed significant inhibition at higher concentrations compared to the other two cyclotides as depicted in Figure 2.

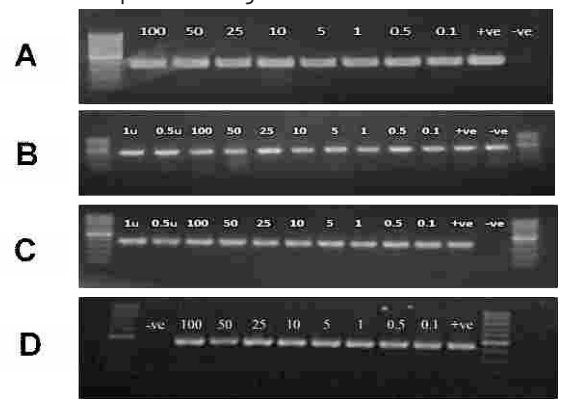


Figure 2: PCR Analysis of NS5A inhibition by cyclotides

Cells were transfected with HCV NS5A and incubated for 24 hrs with cyclotides. The next day total RNA was extracted and cDNA synthesized. This cDNA was used as a template and PCR analysis was conducted using GAPDH as a control. (A) GAPDH PCR (B) Inhibition of NS5A gene by Kalata B12. (C) Inhibition of NS5A gene by Kalata B2. (D) NS5A gene expression in response to Cycloviolacin O14 treatment.

Kalata B12 significantly inhibited HCV NS5A gene

NS5A expression in response to Kalata B12 was quantitatively determined through Real-time PCR. Kalata B12 showed significant inhibition of NS5A at the four highest concentrations from 100nM to 10nM. 100nM showed approximately 75% inhibition of NS5A. Whereas, 50nM, 25nM and 10nM exhibited almost 59%, 50% and 25% inhibition respectively. The graphical representation of the results is shown in Figure 3.

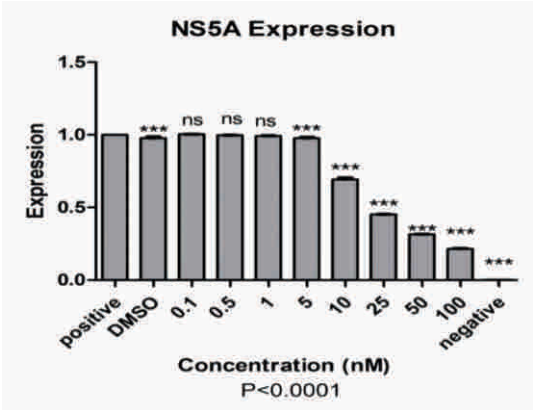


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Figure 3: Effect of KB12 on NS5A expression

Cells were transfected with HCV NS5A and incubated with Kalata B12 cyclotides for 24hrs. Total RNA was extracted and subjected to cDNA synthesis followed by Real-time PCR analysis using GAPDH as an internal control. The data is represented as mean±SD (n=3, One way ANOVA, *p-value <000.1).

DISCUSSION

Cyclotides are an appealing family of peptides derived from plants. These plant peptides contain a wide variety of biological activities such as anti-bacterial, antiviral, insecticidal, cytotoxic, and hemolytic activity . In the present study synthetic peptides are used against HCV. We selected 30 cyclotides from literature with wide pharmacological and biological properties and checked for their molecular interaction with HCV NS5A protein by using docking software, MOE. Molecular docking is a frequently used process of rational drug designing (RDD) that helps to predict the strength of interaction and binding affinity and activity between drug molecules and the target site. Docking also suggests the preferred orientation between two molecules to form a stable complex . Herein, in silico docking studies were conducted between these cyclotides and HCV NS5A protein using MOE software. We selected three cyclotides for in-vitro studies showing strong binding affinity with NS5A. The Kalata B2, Kalata B12, and cycloviolacin O14 showed the binding affinity of -11 Kcal/mol, -9.80 Kcal/mol, and -8.4 Kcal/mol respectively. HCV NS5A is a multifunctional protein that plays a crucial role in viral replication. Thus, literature shows that by targeting this protein HCV replication can be interrupted .

HepG2 cells are cancerous cells derived from a patient of hepatocellular carcinoma. They have high proliferation rate and used in cytotoxicity and drug metabolism studies . MTT Assay is a widely used method in first drug screening to determine cell viability and cytotoxicity in cell lines . Thus, to check cytotoxic potential of these peptides MTT assay was performed on HepG2 cell lines. All three peptides depicted no significant difference in %cell viability as compared to control and showed more than 85% cell viability at concentration from 0.05nM to 1µM. This interprets that these cyclotides are non-cytotoxic and safe for further studies. In a study, peptides showed no significant difference in cell viability in MDCK cell line in control and peptide treated group . After confirmation of cytotoxicity downstream inhibition assay against HCV-NS5A gene was performed. Semi-quantitative PCR analysis was used for expression study of NS5A gene in cells when treated with three cyclotides separately. Kalata B2 and cycloviolacin O14 showed moderate inhibitory effect against NS5A but Kalata B12 showed approx. 75% inhibition of NS5A gene at 100nM concentration. Further Real time PCR analysis was also performed for Kalata B12 and interprets the strong dose dependent inhibition of HCV NS5A. Similarly, cyclotides isolated from Melicope pteleifolia showed potent antiviral activity against influenza A Virus –. In another study, a novel peptide NDFRSKT showed potent antiviral activity against avian influenza virus H9N2 in in vitro and in vivo models . These studies support that cyclopeptide could have potential to be used as antiviral agents.

CONCLUSION

In-silico study can be time saving approach to narrow the range of compounds for potent target screening. Kalata B12 can be a promising potent therapeutic agent against HCV replication. These cyclotides should be further explored against antiviral activity so in future potent antiviral drugs with less side effects may be discovered.

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