

Synthesis, docking and anticancer activity studies of D-proline-incorporated wainunuamide

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Abstract. D-proline-incorporated wainunuamide — a cyclic octapeptide was synthesized and characterized by FTIR, ¹H and ¹³C NMR and Mass spectral analysis. Molecular docking studies were carried out for the designed cyclic octapeptide and the results showed greater affinity for HPV18-2IOI receptor (HeLa cancer cell line). The synthesized cyclic octapeptide exhibited potent anticancer activity against HeLa cancer cells.

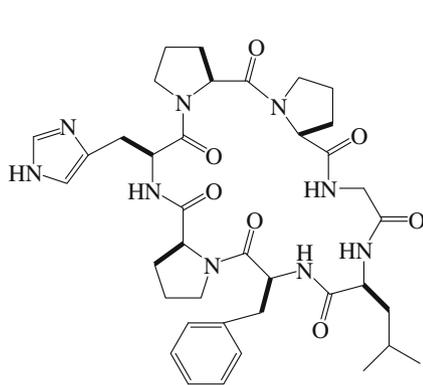
Keywords. Cyclic octapeptide; molecular docking; solution phase synthesis; anticancer activity.

1. Introduction

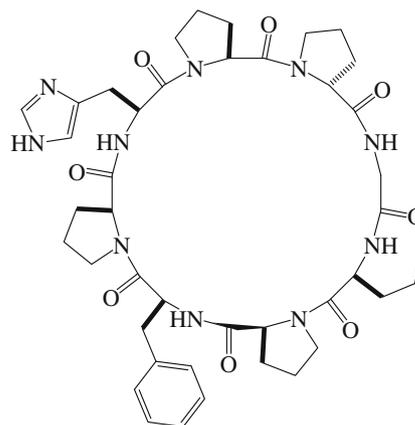
Various active anticancer agents are derived from marine sponges, plants and terrestrial microorganisms. They are rich sources of bioactive cyclic peptides and depsipeptides with unique structures involving a wide variety of unusual amino acids and other building blocks.^{1–4} Many cyclic peptides, isolated from marine sponges, exhibited interesting biological properties including a reverse of multi-drug resistance in tumour cells,⁵ HIV inhibition,^{6,7} and nematocidal activity.⁸

A cyclic heptapeptide, wainunuamide Cyclo-L-[Phe-Pro-His-Pro-Pro-Gly-Leu], was isolated from a *Fijian* marine sponge of *Stylotella aurantium* which showed

moderate cytotoxic activity.⁹ The synthetic wainunuamide also exhibited moderate anticancer activity towards HeLa cancer cell lines.¹⁰ Literature review on cytotoxic cyclic peptides revealed that phakellistatins¹¹ and theonellamides¹² containing D- and/ (or) L- proline units were reported to show potent anticancer activity towards various cancer cells. Based on these results, in order to further enhance the anticancer activity of wainunuamide, a D-proline unit was incorporated into the ring structure of wainunuamide. The cyclic octapeptide Cyclo-D-Pro-(L-Phe-Pro-His-Pro-Pro-Gly-Leu) was synthesized by solution phase peptide technique using TBTU [*O*-(Benzotriazol-1-yl)-*N,N,N,N*-tetramethyluronium tetrafluoroborate] as the coupling reagent and triethylamine as the base.



Wainunuamide



D-Proline incorporated wainunuamide

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2. Experimental

All the reactions requiring anhydrous condition were conducted in flame dried apparatus. Solvents and reagents were purified by standard methods. Organic extracts were dried over anhydrous sodium sulphate. All amino acids and other chemicals were obtained from Spectrochem Private Limited (Mumbai, India). Boc-amino acids and Amino acid methyl ester hydrochlorides were prepared using standard procedures.¹³ Purity of the compounds was checked by pre-coated TLC plate. IR spectra were recorded on Thermo Nicolet FTIR 330 spectrometer using a thin-film supported on KBr pellets. ¹H and ¹³C NMR spectra were recorded on Bruker AC NMR spectrometer using CDCl₃ as solvent. HRMS Mass spectra were recorded on a Jeol GC Mate. Elemental analysis (C, H and N) was carried out using a PERKIN-ELMER 2400 elemental analyzer.

2.1 Preparation of dipeptides

Amino acid methyl ester hydrochloride (10 mmol) was dissolved in chloroform (20 ml). To this, triethylamine (4 ml, 28.7 mmol) was added at 0°C and the reaction mixture was stirred for 15 min. Boc-amino acid (10 mmol) in CHCl₃ (20 ml) and TBTU (10 mmol) were added and the reaction mixture was stirred for half an hour. After 30 min, the reaction mixture was filtered and the residue was washed with CHCl₃ (30 ml) and the washings were added to the filtrate. The filtrate was washed with 5% HCl (20 ml), 5% NaHCO₃ (20 ml) and saturated NaCl (20 ml) solutions. The organic layer was filtered, dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to get the synthesized product. The completion of the reaction was monitored by TLC. Boc-Phe-Pro-OMe, Boc-His-Pro-OMe and Boc-Pro-Gly-OMe were prepared in the similar manner.

2.2 Preparation of Boc-Phe-Pro-His-Pro-OMe

The above tetrapeptide was prepared from the dipeptide Boc-Phe-Pro-OMe and Boc-His-Pro-OMe units after appropriate deprotection¹⁴ at the required functional groups. The deprotected dipeptide units were coupled using TBTU, Et₃N to get the protected tetrapeptide using the procedure similar to that of the dipeptide.

2.2a Tetrapeptide: Boc-Phe-Pro-His-Pro-OMe: Brown sticky semisolid mass, Molecular weight: 610, Molecular Formula: C₃₁H₄₂N₆O₇, Yield 79%; UV (100% MeOH) λ_{max} 249; FTIR (KBr γ cm⁻¹): 3227 (-NH Stretch), 2982 and 2964 (-CH Stretch), 1749 (>CO

of ester group), 1681 (>CO Stretch), 1570 (-NH Bend), 1461 (-CH bend). ¹H NMR (500 MHz, CDCl₃): δ 8.0–7.5 (m, 7H, Ar-H), 7.2 (s, 1H, -NH of imidazole), 7.0 (s, 1H, -NH of Phe), 4.8 (t, 2H, α-CH of Phe and His), 4.2 (t, 2H, N-CH of Pro), 4.0 (d, 2H, -CH₂ of Phe and His), 3.8 (s, 3H, -OCH₃), 3.2–2.4 (m, 8H, -CH₂ of Pro), 3.4–3.3 (t, 4H, N-CH₂ of Pro), 0.9 (s, 9H, -CH₃ of Boc group). HRMS (EI) m/z: 610.61 (M⁺). Anal. Calcd. for C₃₁H₄₂N₆O₇: C 60.97, H 6.93, N 13.76. Found: C 60.89, H 6.80, N 13.66.

2.3 Preparation of Boc-Pro-Gly-Leu-OMe

The above tripeptide was prepared from the dipeptide Boc-Pro-Gly-OMe and Leu-OMe units after appropriate deprotection¹⁴ at the required functional groups. The deprotected dipeptide and Leu-OMe units were coupled using TBTU, Et₃N to get the protected tripeptide using the procedure similar to that of the dipeptide.

2.3a Tripeptide: Boc-Pro-Gly-Leu-OMe: Pale Yellowish sticky semisolid mass, Molecular weight: 399, Molecular Formula: C₁₉H₃₃N₃O₆, Yield 77%; UV (100% MeOH) λ_{max} 232; FTIR (KBr γ cm⁻¹): 3221 (-NH Stretch), 2979 and 2960 (-CH Stretch), 1746 (>CO of ester group), 1680 (>CO Stretch), 1576 (-NH Bend), 1466 (-CH bend). ¹H NMR (500 MHz CDCl₃): δ 7.5–7.7 (s, 2H, -NH), 4.1 (t, 1H, N-CH of Pro), 3.9 (t, 1H, -CH of Leu), 3.6 (s, 3H, -OCH₃ of Leu), 3.4–3.2 (m, 2H, -CH₂ of Pro), 3.5–3.0 (t, 2H, N-CH₂ of Pro), 2.9–2.5 (m, 2H, -CH₂ of Pro), 1.8 (d, 2H, -CH₂ of Leu), 1.7 (m, 1H, -CH of Leu), 1.4 (s, 2H, -CH₂ of Gly), 1.0 (s, 6H, -CH₃ of Leu), 0.9 (s, 9H, -CH₃ of Boc group). HRMS (EI) m/z: 399.00 (M⁺). Anal. Calcd. for C₁₉H₃₃N₃O₆: C 57.12, H 8.33, N 10.52. Found: C 57.04, H 8.25, N 10.42.

2.4 Preparation of Boc-D-Pro-Phe-Pro-His-Pro-OMe

The above pentapeptide was prepared from the tetrapeptide Boc-Phe-Pro-His-Pro-OMe and Boc-D-Proline units after appropriate deprotection¹⁴ at the required functional groups. The deprotected tetrapeptide and Boc-D-Proline units were coupled using TBTU, Et₃N to get the protected pentapeptide using the procedure similar to that of dipeptide.

2.5 Preparation of linear octapeptide - Boc-D-Pro-L-(Phe-Pro-His-Pro-Pro-Gly-Leu-OMe)

The Boc-group of the tripeptide (Boc-Pro-Gly-Leu-OMe) was removed and the ester group of the

pentapeptide (Boc-D-Pro-Phe-Pro-His-Pro-OMe) was deprotected. Both the deprotected units were coupled to get the linear octapeptide- Cyclo D-Pro-L-(Phe-Pro-His-Pro-Pro-Gly-Leu).

2.6 Preparation of Cyclo-D-Pro-L-(Phe-Pro-His-Pro-Pro-Gly-Leu)

The cyclisation of the linear octapeptide unit was carried out by *p*-nitrophenyl ester method of Bodanszky¹⁵ with certain modifications. The ester group of the linear segment was removed with LiOH and the *p*-nitrophenyl ester group was introduced using the following procedure:

To the solution of 2.2 mmol of Boc-peptide carboxylic acid in 15 ml of CHCl₃ was added and 0.31 ml (2.2 mmol) of triethylamine. After cooling in an ice bath, 0.44 g (2.2 mmol) of *p*-nitrophenol¹⁶ was added and stirred for 12 h at room temperature. The reaction mixture was filtered and the filtrate was washed with 10% NaHCO₃ (5 ml) solution until excess of *p*-nitrophenol was removed and finally washed with 5% HCl (5 ml) to get Boc-Peptide-pnp-ester.

To the above Boc-peptide-pnp-ester (2 mmol) in CHCl₃ (10 ml), CF₃COOH (2 mmol) was added, stirred for 1 h at room temperature and washed with 10% NaHCO₃ solution. The organic layer was dried over anhydrous Na₂SO₄. To the Boc-deprotected peptide-pnp-ester (1.5 mmol) in CHCl₃ (10 ml), TBTU (1.5 mmol), triethyl amine (1.5 mmol) was added and stirred for half an hour. The reaction mixture was washed with 10% NaHCO₃ until the byproduct *p*-nitrophenol was removed completely and finally washed with 5% HCl (5 ml) dried and evaporated *in vacuo* to get the cyclised product Cyclo-D-Pro-L-(Phe-Pro-His-Pro-Pro-Gly-Leu).

2.6a Cyclic octapeptide: Cyclo-D-Pro-L-(Phe-Pro-His-Pro-Pro-Gly-Leu): Yellowish semisolid mass, Molecular weight: 843, Molecular Formula: C₄₃H₅₈O₈N₁₀, Yield 75%; UV (100% MeOH) λ_{max} 300; FTIR (KBr γ cm⁻¹): 3331 (-NH Stretch), 2989 and 2964 (-CH Stretch), 1716 (CO Stretch), 1569 (-NH Bend), 1458 (-CH bend). ¹H NMR (500 MHz CDCl₃): δ 7.8–7.1 (m, 7H, Ar-H), 7.3 (s, 1H, -NH of imidazole), 7.2–6.8 (s, 3H, -NH), 4.7 (t, 2H, -CH of Phe and His), 4.3 (t, 4H, N-CH of Pro), 4.0 (d, 4H, -CH₂ of Phe and His), 3.0–2.7 (t, 8H, N-CH₂ of Pro), 3.8 (t, 1H, -CH of Leu), 3.5–3.2 (m, 16H, -CH₂ of Pro), 1.8 (d, 2H, -CH₂ of Leu), 1.7 (m, 1H, -CH of Leu), 1.4 (s, 2H, -CH₂ of Gly), 1.0 (s, 6H, -CH₃ of Leu). ¹³C NMR (125 MHz CDCl₃): δ 168.9 (>CO of Phe), 170.5 (>CO

of Gly), 171.3 (>CO of His), 172.1 (>CO of Pro), 173.8 (>CO of Leu), 174.7 (>CO of Pro), 175.2 (>CO of Pro), δ 119.2, 127.4, 128.4, 130.3, 130.5, 134.9, 138.7, (Ar-C of Phe and His), δ 60.6 (Pro α-CH₂), 61.4 (Pro α-CH₂), 62.6 (Pro α-CH₂), 64.3s (Pro α-CH₂), δ 53.5 (His α-CH), δ 50.7 (α -CH of Phe), 44.8 (Gly α-CH₂), δ 41.5 (Leu -CH₂), δ 45.1 (Pro δ-CH), 46.7 (Pro δ-CH), 46.9 (Pro δ-CH), 47.2 (Pro δ-CH), δ 37.7 (α-CH₂ of Phe), 28.7 (Pro β-CH₂), δ 29.1 (Pro β-CH₂), 32.4 (Pro β-CH₂), 32.7 (Pro β-CH₂), δ 28.7 (His β-CH₂) δ 22.7 (Pro γ-CH₂), 24.3 (Pro γ-CH₂), 24.8 (Pro γ-CH₂), 25.2 (Pro γ-CH₂), δ 20.5 and 20.8 (Leu α 2-CH₃). HRMS (EI) m/z: 843.17 (M⁺). Anal. Calcd. for C₄₃H₅₈O₈N₁₀: C 61.27, H 6.93, N 16.62. Found: C 61.19, H 6.79, N 16.52.

2.7 Molecular docking

We have used the following bioinformatics tools; biological databases like PDB (Protein Data Bank),^{17,18} Swiss-PDB Viewer Version¹⁹ 3.7 and docking software - Hex Version²⁰ 5.1 ACD/ChemSketch (ACD/Labs, www.acdlabs.com). The Protein Data Bank (PDB) is the single worldwide archive of structural data of biological macromolecules, established in Brookhaven National Laboratories.²¹ Computer aided drug design methods are heavily dependent on bioinformatics tools, applications and databases.²² The structure of 2IOI (HPV - human papillomavirus) receptor molecule (figure 1) was retrieved from protein data bank (PDB Code: 2IOI).

Using ChemSketch the structure of the cyclic octapeptide was sketched. The docking analysis of the cyclic octapeptide with 2IOI was carried by HEX docking software. Docking allows the scientist to virtually screen a database of compounds and predict the strongest binders based on the various scoring functions. It explores ways in which two molecules, such as ligand and 2IOI receptor fit together and dock to each other. The cyclic octapeptide molecules binding to a 2IOI receptor, inhibit its function, and thus act as an anticancer drug. The collection of drug and receptor complex was identified via docking and their relative stabilities were evaluated using molecular dynamics and their binding affinities, using free energy simulations. Based on the total energy values, the anticancer activity of the compound was identified.

2.8 Anticancer activity

MTT [3-(4, 5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide] assay^{23,24} - (Human breast adenocarcinoma cell line) were obtained from ATCC and

Table 1. Docking results of cyclic octapeptide with different anticancer receptors.

S. No	(PDB Code)	Cyclic octapeptide E total (kJ/mol)
1	1ZZF	-119.14
2	1JLM	-108.96
3	1IDO	-73.14
4	1R8P	-68.17
5	2IOI	-160.11

Standard anticancer drug — Camptothecin -141.65 (kJ/mol).

maintained in DMEM (Hi-Media Laboratories Pvt. Ltd, Mumbai, India) supplemented with 10% heat-inactivated FBS (v/v), streptomycin (100 µg/ml) and penicillin (100 µg/ml). The cell line was maintained at 37°C with 5% carbon dioxide in CO₂ incubator. The MTT cell proliferation assay was used to evaluate the anticancer activity of D-Proline incorporated wainunuamide using the Cell Quantification MTT cell viability assay kit (Bioassay Systems). The optical density was measured at 570 nm for each well on the absorbance plate reader. Trypan blue dye exclusion assay was also used to count the number of viable and nonviable HeLa cancer cells in the culture medium after drug treatment. Treatment with 5-FU at the same concentration served as positive control.

3. Results and discussion

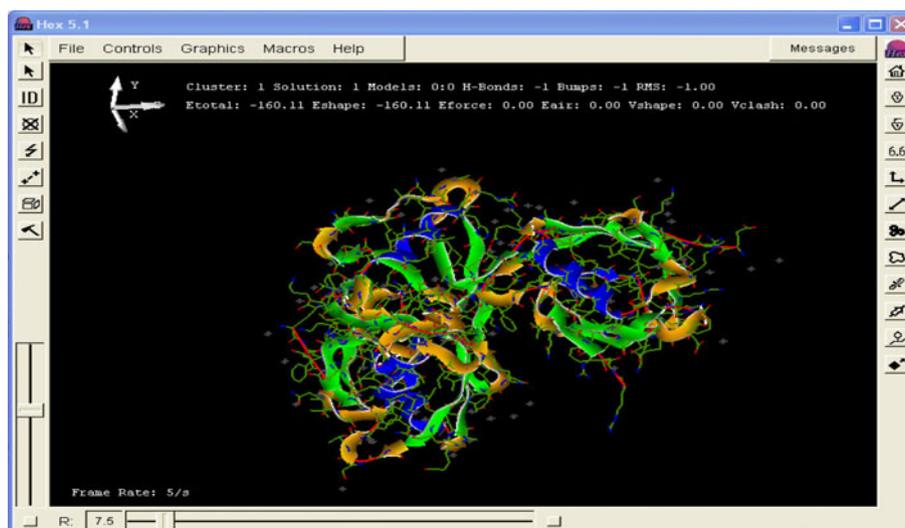
In order to carry out the synthesis, the wainunuamide was disconnected into one tripeptide and tetrapeptide

units, Boc-Pro-Gly-Leu-OMe and Boc-Phe-Pro-His-Pro-OMe. Both tripeptide and tetrapeptide units were synthesized by standard method using TBTU as coupling reagent.²⁵ The synthesized tetrapeptide was treated with Boc-D-Proline to get pentapeptide unit. The tripeptide and pentapeptide units containing Boc- and ester groups were properly deprotected and coupled together to get the linear octapeptide. Finally the linear octapeptide was cyclised using *p*-nitrophenyl ester method (scheme 1).

FTIR spectrum of cyclic octapeptide showed characteristic medium to strong bands corresponding to the carbonyl stretching at 1716 cm⁻¹ and NH bending at 1569 cm⁻¹, confirmed the coupling reaction of the cyclic octapeptide. ¹H and ¹³C NMR spectrum of the synthesized cyclic octapeptide showed a peak corresponds to the peptide clearly confirm the coupling of amino acids and peptides. The mass spectral data confirm a stable molecular ion peak [843.17 - M⁺] for the synthesized compound. Based on the above mentioned spectral datas, the structure of the cyclic octapeptide was confirmed. The spectral data values for the synthesized cyclic octapeptide are shown in the experimental section.

Docking studies have been carried out using Hex software tool. The designed cyclic octapeptide were docked with different target cancer receptors collected from protein database bank, out of all receptors HPV18-2IOI receptor had shown good binding interaction with the (ligand) cyclic octapeptide. The results were shown in table 1.

The D-Proline incorporated wainunuamide had shown good docking score [-160.11 kJ/mol] (figure 2) with HPV18-2IOI proteins. The docking result was

**Figure 2.** Interaction and binding energy of cyclic octapeptide with 2IOI receptor.

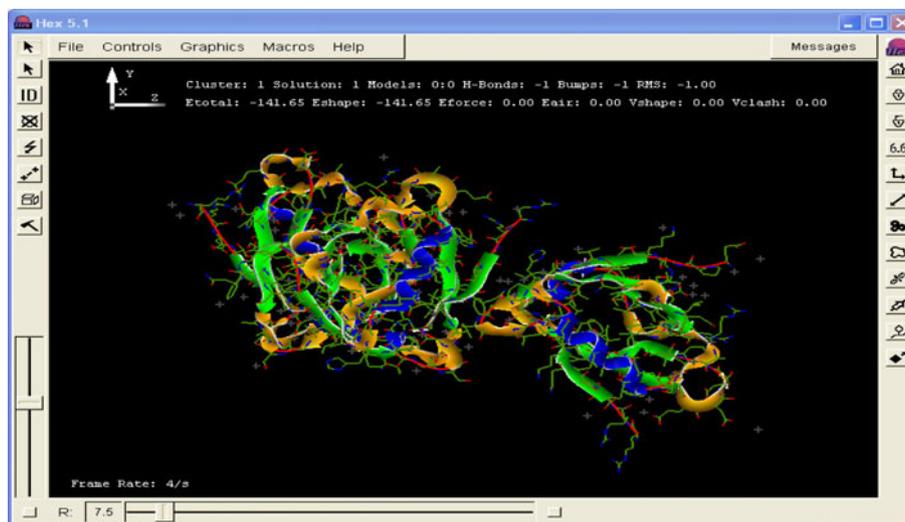
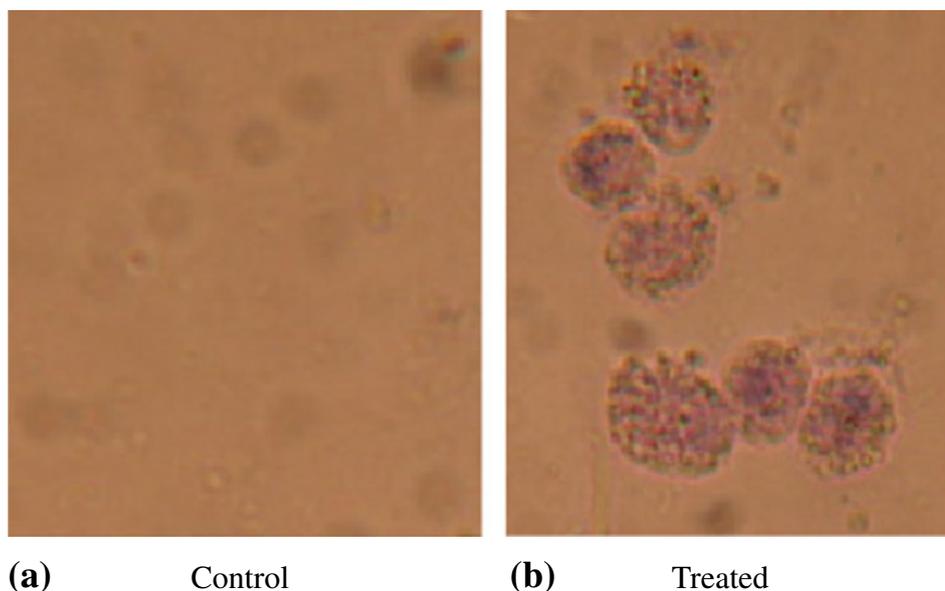


Figure 3. Interaction and binding energy of camptothecin with 2IOI receptor.



(a) Control

(b) Treated

Figure 4. Representative phase micrograph of HeLa cancer cell lines of control group and of those treated with the one studied preparations **(a)** Control cells, untreated HeLa cells, (100% viability); **(b)** HeLa cells treated with cyclic octapeptide (**1**) at 50 µg/ml (27% viability).

compared with that of standard anticancer drug Camptothecin [-141.65 kJ/mol] (figure 3). Based on the docking score, the synthesized compound was evaluated for the anticancer activity towards HeLa cancer cell line.

The effects of D-Proline incorporated cyclic octapeptide on the growth of HeLa cancer cells were tested under *in vitro* conditions using different concentrations (5, 15, 25 and 50 µg/ml) and the cell survival after 48 h was shown in the figure 4. The D-Proline incorporated wainunuamide inhibited the growth of HeLa cancer cells and the results are shown in the table 2. The results

of D-Proline-incorporated wainunuamide were compared with synthetic wainunuamide. The D-proline unit containing wainunuamide showed potent anticancer activity than that of synthetic wainunuamide. The IC_{50} values were calculated to be 31.75 µg/ml for D-Proline incorporated wainunuamide and 20.58 µg/ml for synthetic wainunuamide. After 96 h, D-Proline-incorporated wainunuamide (50 µg/ml) exhibited potent anticancer activity 27% (73% cell death) on HeLa cancer cells, but the synthetic wainunuamide had shown 53% cell viability (47% cell death) on HeLa cancer cells.

Table 2. Effect of cyclic octapeptide on HeLa cancer cell line.

Anticancer activity of D-Proline incorporated cyclic heptapeptide (wainunuamide) on HeLa cell lines after 48 h treatment ^a		
Compound	Percentage of viable cells	
	Control	Treated
Cyclic octapeptide	100	27

^aValues are mean of the three experiments. The viable HeLa cells were calculated after 48 h of cyclic octapeptide treatment and strained with trypan blue dye exclusion test

4. Conclusion

In conclusion, TBTU was found to be effective coupling reagent giving high yields and reduced reaction time (30 min) as compared to DCC (24 h) and EDC (12 h). Molecular docking studies concluded that the designed cyclic octapeptide showed greater affinity towards HPV18-2IOI target protein for cancer. Based on the docking results, the synthesized cyclic octapeptide was evaluated for the anticancer activity and the compound showed potent anticancer activity as compared to that of the parent cyclic heptapeptide — wainunuamide.

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