Research Article

Docking, Synthesis and Anticancer Activity of Metallo Peptides Using Solution Phase Peptide Technique

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ABSTRACT

The present work deals with synthesis and characterization of the ligand dipeptide and their complexes with Ni (II) and Cu (II) ions. The synthesized dipeptide complexes have been characterized by UV-Visible, FTIR, ^{1}H & ^{13}C NMR and Mass spectral analysis. Molecular docking studies were carried out for the Metal incorporated Linear peptides and the results showed greater affinity for HPV18-2IOI receptor (HeLa cancer cell line). Metal incorporated linear peptides [*5(a-d) & 6(a-d)*] showed potent anticancer activity against HeLa cancer cells.

Keywords: Synthesis, Ni (II) and Cu (II) complexes, docking, solution phase peptide synthesis, anticancer activity.

INTRODUCTION

Metal ion containing complexes plays an important role in the biological system of the living organisms[1]. The main medicinal uses and application of metals and metal containing complexes are of increasing in clinical and commercial importance[2]. In general metal ion containing compounds possess a variety of biological activities like antibacterial and antifungal[3], antithrombotic[4], antiulcerous[5] and anticancer activities[6]. An extensive literature survey was done in the synthesis and biological evaluation of metal incorporated linear and cyclic peptide compounds. Survey revealed that only a few articles were available for metal incorporated peptides synthesis by solid phase peptide technique[7]. The disadvantages of solid phase technique were (i) the cost of solid support material is expensive (ii) protection and deprotection of solid resin is very difficult (iii) stability of the complexes is weak. By keeping in view of these observations, we have tried the synthesis of metal incorporated linear peptides using solution phase technique.

MATERIALS AND METHODS

IR spectra were recorded on Thermo Nicolet 330 FTIR spectrophotometer (Thermonicolet, USA) using a thin film supported on KBr pellets. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AC NMR spectrometer (300 MHz), (Bruker, USA) using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. Mass spectra were recorded in Joel Sax 102/DA-6000 mass spectrometer (Joel, Tokyo, Japan) operating at 70 eV using fast atom bombardment technique. All amino acids and other chemicals were obtained from Spectrochem Private Limited (Mumbai, India).

Preparation of amino acid methyl ester hydrochlorides

Thionyl chloride (1.4ml, 20mmol) in methanol (100ml) added slowly at 0°C. To the resulting solution, amino acid (20mmol) was added and refluxed for about 8-10 hours. The excess solvent was evaporated to get the amino acid methyl ester hydrochloride which was triturated with diethyl ether at 0°C until excess dimethyl sulphite was removed. The resulting solid was recrystallized from methanol and diethyl ether at 0°C. Using the above procedure, the following amino acid methyl ester hydrochlorides were prepared - (i) Glycine OMe.HCl, (ii) Tyrosine OMe.HCl, (iii) Leucine OMe.HCl, (iv) Alanine OMe.HCl. The physical data of the synthesized amino acid methyl esters are illustrated in the Table 1.

Preparation of Boc-amino Acids

The amino acid (20mmol) was dissolved in 1N NaOH (20ml) and isopropanol (20ml). Tert-butyloxy carbonyl anhydride $(Boc)_2O$ (26mmol, 6ml) in isopropanol (10ml) was added followed by 1N NaOH (20ml) to the resulting solution. The solution was stirred at room temperature for 2 hours, washed with light petroleum ether (40-60°C) (20ml), acidified to pH 3.0 with 2N H_2SO_4 and finally extracted with chloroform (3 x 20ml). The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure to get Boc-amino acids. The crude product was recrystallized using chloroform and petroleum ether as solvents. Using the above method the following Boc-amino acids were prepared - (i) Boc-glycine, (ii) Boc-alanine. The physical parameters of the synthesized Boc-amino acids are mentioned in the Table 2.

Preparation of the Dipeptides[8]

Amino acid methyl ester hydrochloride (10mmol) was dissolved in chloroform (20ml). To this, N-methylmorpholine (1.3ml) was added at 0°C and the reaction mixture was stirred for 15 minutes. Boc-amino acid (10mmol) in $CHCl_3$ (20ml) and TBTU (10mmol) were added with stirring. After 30 minutes, the reaction mixture was filtered. The filtrate was washed with 5%

$NaHCO_3$ (20ml), 5% HCl (20ml) and distilled H_2O (20ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated in a vacuum. The residue was purified by recrystallization from $CHCl_3$. The ester and Boc-group were deprotected using standard procedure (*A&B*).The following dipeptides was prepared using the above mentioned protocol: (i) Glycyl-glycine; (ii) Glycyl-tyrosine; (iii) Alanyl-leucine; (iv) Alanyl-alanine. The physical data of the synthesized dipeptides are represented in the Table 3.

Table 1: Physical Data of the synthesized amino acid methyl esters

S.No	Amino acid methyl ester	Molecular Weight	Molecular Formula	M.P (°C) - (Lit. M.P)	% Yield
1	Glycine methyl ester hydrochloride	125.55	$C_3H_8ClNO_2$	175 (173-177)	74.98
2	Tyrosine methyl ester hydrochloride	231.07	$C_{10}H_{13}NO_3HCl$	187 (185-191)	97.76
3	Leucine methyl ester hydrochloride	181.66	$C_7H_{15}NO_2HCl$	151 (151-153)	74.80
4	Alanine methyl ester hydrochloride	139.58	$C_4H_9NO_2HCl$	110 (109-111)	77.82

Table 2: Physical Data of the synthesized Boc-amino acids

S.No	Boc-amino acids	Molecular Weight	Molecular Formula	M.P (°C) – (Lit. M.P)	% Yield
1	Boc-Glycine	175.18	$C_7H_{13}NO_4$	87 - (86-89)	81.75
2	Boc-Alanine	189.21	$C_8H_{15}NO_4$	81 - (79-83)	88.90

Table 3: Physical Data of the synthesized dipeptides

Boc-dipeptide- OMe	Physical state	Molecular formula	Molecular weight	% yield
Boc-Gly-Gly-OMe	Colourless semisolid mass	$C_{10}H_{18}N_2O_5$	246.12	83.23
Boc-Gly-Tyr-OMe	Pale yellow semisolid mass	$C_{17}H_{24}N_2O_6$	352.16	78.72
Boc-Ala-Leu-OMe	Colourless semisolid mass	$C_{15}H_{28}N_2O_5$	316.20	81.02
Boc-Ala-Ala-OMe	Colourless Semisolid mass	$C_{12}H_{22}N_2O_5$	274.15	79.14

Deprotection of ester group: To a solution of the protected peptide (1mmol) in THF: H_2O (1:1) (36ml), LiOH (1.5mmol) was added at 0°C. The mixture was stirred for about 1hour at room temperature and then acidified to pH 3.5 with 1N H_2SO_4. The aqueous layer was extracted with Et_2O (3 x 15ml). The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure.

Deprotection of amino group: The protected peptide (1mmol) was dissolved in $CHCl_3$ (15ml) and treated with CF_3COOH (2mmol, 0.228gms). The solution was stirred at room temperature for about 1hour, washed with saturated $NaHCO_3$ (5ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The product was purified by recrystallization from $CHCl_3$ and petroleum ether.

Preparation of the metal complexes

Complexes of Ni(II) and Cu(II) ions with dipeptides were prepared by mixing ethanolic solutions (40mL) of 0.01mmol of the synthesized ligand (dipeptides) with an ethanolic solution (40mL) of 0.01mmol of $NiCl_2.6H_2O$ and $CuCl_2.2H_2O$ respectively, and a few drops of sodium bicarbonate solution were added to adjust the pH-8.5 until the complexes gets isolated. The reaction mixtures were refluxed for three hours and then cooled & filtered by suction process. The completion of the reaction was monitored by TLC. The metal complexes were dried in desiccators over anhydrous calcium chloride under vacuum. The following metallopeptides were prepared using the above mentioned protocol: *Cu(II)-glycyl-glycine*, *Ni(II)-glycyl-glycine*, *Cu(II)-glycyl-tyrosine*, *Ni(II)-glycyl-tyrosine*, *Cu(II)-alanyl-leucine*, *Ni(II)-alanyl-leucine*, *Cu(II)-alanyl-alanine*, *Ni(II)-alanyl-alanine*.

Spectral Data:

Copper(II)-glycyl-glycine ***5(a):*** Brown solid, Molecular Formula: $C_4H_6CuN_2O_3$; Molecular weight: 192.97; FTIR (υ, KBr) cm^{-1}: 538.18 (Cu-N); 642.70 (Cu-O).^{1}H NMR (500 MHz, D_2O): δ 4.41 (s, 2H, -CH_2 of Gly), 3.54 (s, 2H, -CH_2). ^{13}C NMR (125 MHz, D_2O): δ 170.8, 168.9 (>C=O of Gly), 38.2, 40.6 (-CH_2 of Gly). HRMS (EI): 192.9701 (M+).

Nickel(II)-glycyl-glycine ***6(a):*** Pale green solid, Molecular Formula: $C_4H_6NiN_2O_3$; Molecular weight: 187.97; FTIR (υ, KBr) cm^{-1}: 541.23 (Ni-N); 636.15 (Ni-O).^{1}H NMR (400 MHz, DMSO-d^6): δ 4.40 (s, 2H, -CH_2 of Gly), 3.52 (s, 2H, -CH_2), 9.48 (broad, 1H, NH), 5.59 (s, 1H, -NH); HRMS (EI): 187.1024 (M+).

Copper(II)-glycyl-tyrosine ***5(b):*** Brown solid, Molecular Formula: $C_{11}H_{12}CuN_2O_4$; Molecular weight: 299.01; FTIR (υ, KBr) cm^{-1}: 536.04 (Cu-N); 646.08 (Cu-O).^{1}H NMR (400

MHz, D_2O): δ 7.02 (d, 2H, Ar-H of Tyr), 6.68 (d, 2H, Ar-H of Tyr), 4.43 (s, 2H, -CH_2 of Gly), 3.95 (t, -1H, -CH of Tyr), 3.19 (d, 2H, -CH_2 of Tyr). ^{13}C NMR (125 MHz, D_2O): δ 177.9, 166.17 (>C=O of Gly and Tyr), 131.6-115.3 (Ar-C of Tyr), 56.7 (-CH of Tyr), 36.6, 40.3 (-CH_2 of Gly). HRMS (EI): 299.1802 (M+).

Nickel (II)-glycyl-tyrosine ***6(b):*** Pale green solid, Molecular Formula: $C_{11}H_{12}NiN_2O_4$; Molecular weight: 294.02; FTIR (υ, KBr) cm^{-1}: 536.14 (Ni-N); 644.51 (Ni-O). ^1H NMR (400 MHz, D_2O): δ 7.03 (d, 2H, Ar-H of Tyr), 6.69 (d, 2H, Ar-H of Tyr), 4.42 (s, 2H, -CH_2 of Gly), 3.96 (t, -1H, -CH of Tyr), 3.17 (d, 2H, -CH_2 of Tyr). HRMS (EI): 294.1923 (M+).

Copper(II)-alanyl-leucine ***5(c):*** Brown solid, Molecular Formula: $C_9H_{16}CuN_2O_3$; Molecular weight: 263.05; FTIR (υ, KBr) cm^{-1}: 539.08 (Cu-N); 643.21 (Cu-O).^1H NMR (400 MHz, D_2O): δ 4.62 (m, 1H, -CH of Ala), 3.55 (t, 1H, -CH of Leu), 2.47 (m, 1H, -CH of Leu); 2.12 (d, -CH_2 of Leu); 1.01 (d, -CH_3 of Leu), 0.96 (d, -CH_3 of Leu). HRMS (EI): 263.6752 (M+).

Nickel(II)-alanyl-leucine ***6(c):*** Pale green solid, Molecular Formula: $C_9H_{16}NiN_2O_3$; Molecular weight: 258.05; FTIR (υ, KBr) cm^{-1}: 539.06 (Ni-N); 644.31 (Ni-O).^1H NMR (400 MHz, D_2O): δ 4.58 (m, 1H, -CH of Ala), 3.51 (t, 1H, -CH of Leu), 2.46 (m, 1H, -CH of Leu); 2.11 (d, -CH_2 of Leu); 1.04 (d, -CH_3 of Leu), 0.94 (d, -CH_3 of Ala). HRMS (EI): 258.9120 (M+).

Copper(II)-alanyl-alanine ***5(d):*** Brown Solid, Molecular Formula: $C_6H_{10}CuN_2O_3$; Molecular weight: 221.07; FTIR (KBr) cm^{-1}: 527.04 (Cu-N); 642.70 (Cu-O).^1H NMR (400 MHz, D_2O): δ 3.92 (s, 1H, -CH of Ala), 3.62 (s, 1H, -CH of Ala), 1.09 (d, 6H,-CH_3 of Ala). HRMS (EI): 221.9701 (M+).

Nickel(II)-alanyl-alanine ***6(d):*** Brown Solid, Molecular Formula: $C_6H_{10}NiN_2O_3$; Molecular weight: 216.35; FTIR (υ, KBr) cm^{-1}: 537.08 (Ni-N); 643.09 (Ni-O).^1H NMR (400 MHz, D_2O): δ 3.94 (s, 1H, -CH of Ala), 3.63 (s, 1H, -CH of Ala), 1.08 (d, 6H,-CH_3 of Ala). HRMS (EI): 216.1401 (M+).

Molecular Docking

We used the following Bioinformatics tools; biological databases like PDB (Protein Data bank) [9, 10] Swiss-PDB Viewer Version[11] 3.7 and docking software like Hex Version[12] 5.1 ACD ChemSketch (ACD/Labs, *www.acdlabs.com*). The Protein Data Bank (PDB) is the single worldwide archive of structural data of biological molecules, established in Brookhaven National Laboraties[13]. Computer aided drug design methods are heavily dependent on Bioinformatics tools, applications and databases[14]. The structure of 2IOI (HPV - human papillomavirus) receptor molecule was retrieved from protein data bank (PDB Code: 2IOI).

Using ChemSketch the structure of the metallopeptides was sketched. The docking analyses of the metallopeptides with 2IOI were carried by HEX docking software. Docking allows the scientist to virtually screen a database of the 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 Cu (II) and Ni (II) incorporated linear peptides 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 metallopeptides was identified.

Anticancer activity

MTT [3-(4, 5-dimethylthiazole-2-yl)-2,5diphenyl tetrazoliumbromide] Assay[15,16] - (HeLa cell line) were obtained from ATCC and maintained in DMEM (Hi-Media Laboratories Pvt. Ltd, Mumbai, India) supplemented with 10% heat-inactivated FBS (v/v), streptomycin (100µg/ml) and penicillin (100µg/ml). The cell line was maintained at 37°C with 5% carbon dioxide in CO_2 incubator. The MTT cell proliferation assay was used to evaluate the anticancer activity of the Cu (II) and Ni (II) incorporated linear peptides using the Cell Quantification MTT cell viability assay kit (Bioassay Systems). The optical density was measured at 570nm for each well on the absorbance plate reader. Trypan blue dye exclusion assay was also used to count the number of viable and non-viable HeLa cancer cells in the culture medium after drug treatment. Treatment with 5-FU in the same concentration served as positive control.

RESULTS AND DISCUSSION

The metal complexes were synthesized by reaction of the ligand (dipeptide) with the metal ions in 1:2 molar ratios in ethanolic (or) water medium as shown in the reaction **scheme-I**. The ligand behaves as bidentate coordinate through oxygen and nitrogen donor atoms. Infrared spectrum of ligand (glycyl-tyrosine) shows several bands at 3489.22, 3392.65 and 1690.32 cm^{-1} (Figure 1), due to the presence of >OH, NH_2 and >C=O respectively. The infrared spectral data of Cu (II)-glycyl-tyrosine complex showed the disappearance of the band due to the coordination of the NH_2 with the central metal ion. The band at 1690.32 cm^{-1} which attributed to the >C=O in free ligand is shifted to lower frequency 1610.01 cm^{-1} in the spectra of the complexes.

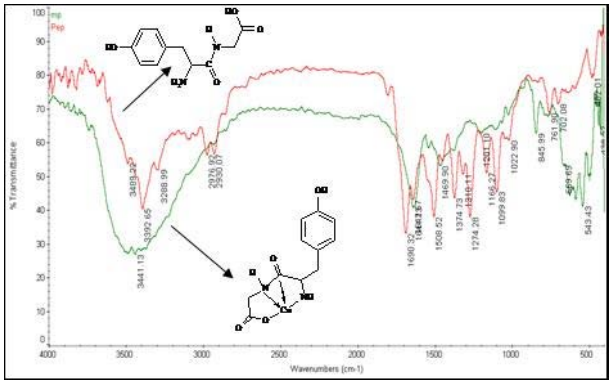
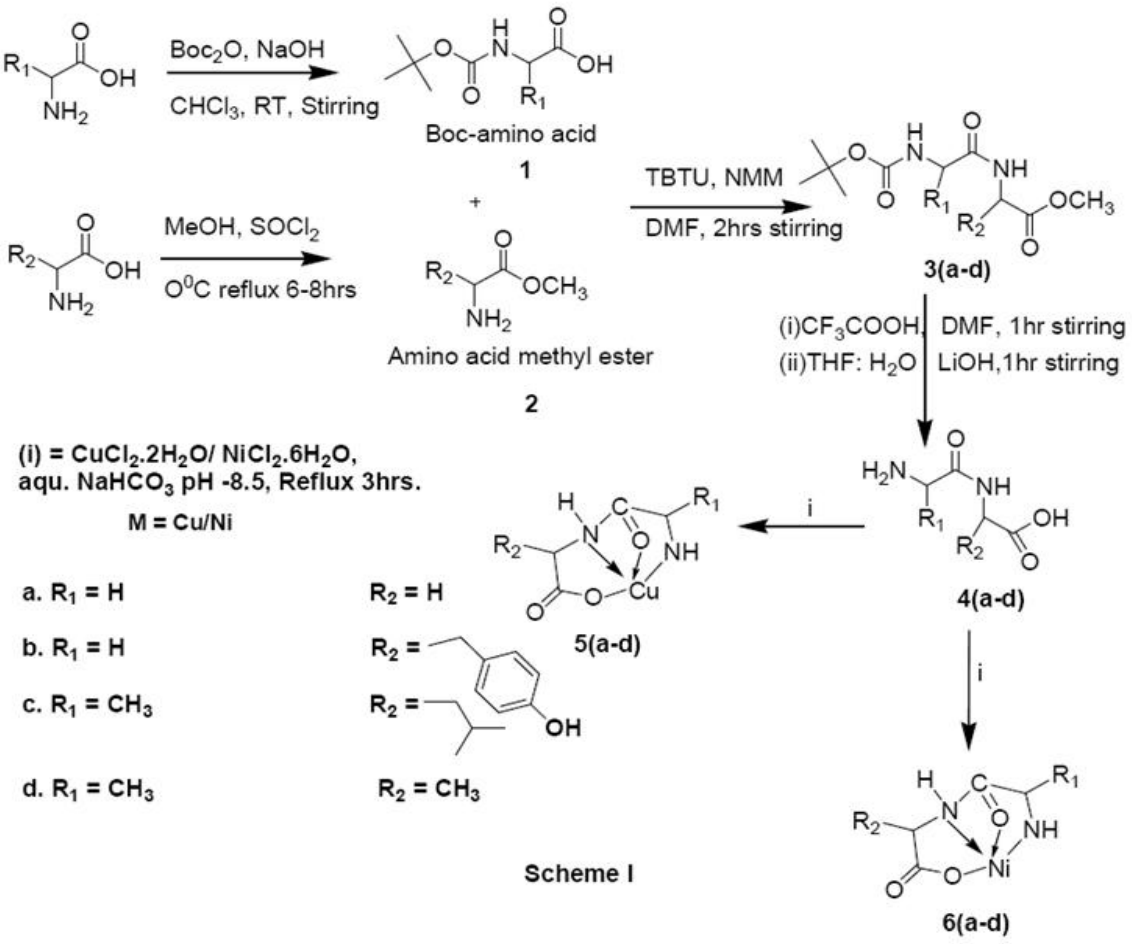


Figure 1: FTIR Spectrum of Copper (II)-glycyl-tyrosine (5b) and glycyl-tyrosine (4b)

Similarly, the -OH band in the free ligand disappeared in the spectra of the complexes due to the coordination with the central metal ion. New bands at 543.43 and 669.69 cm^{-1} endorsed to the existing of (Cu-N) and (Cu-O) vibrations. The appearance of these vibrations which are not present in the free ligand indicates the involvement of nitrogen and oxygen atoms in chelation. The electronic absorption spectra of the ligand displayed a band at 347nm which is due to the phenyl ring of tyrosine unit. Due to d-d and charge transfer transitions, the electronic spectrum of Cu (II) complex showed two bands at 583 and 396nm respectively. The electronic spectral data of the metallopeptides has shown in the table 4.

Table 4: Electronic spectral data of metallopeptides

Metal complexes	D → d transition	CT → M
Cu(II)-glycyl-glycine (**5a**)	539 (0.392)	352 (0.920)
Cu(II)-glycyl-tyrosine (**5b**)	583 (0.594)	347(0.126)
Cu(II)- alanyl-leucine (**5c**)	567 (0.782)	321 (0.391)
Cu(II)-alanyl-alanine (**5d**)	549(0.921)	338 (0.673)
Ni(II)-glycyl- glycine (**6a**)	521 (0.109)	399 (0.916)
Ni(II)-glycyl-tyrosine (**6b**)	543 (0.456)	376 (0.223)
Ni(II)- alanyl-leucine (**6c**)	507 (0.189)	342 (0.092)
Ni(II)- alanyl-alanine (**6d**)	496 (0.789)	331 (0.499)



Scheme I

The ^1H NMR spectra of glycyl-tyrosine showed two distinct doublets at [δ, 2H, d, 7.01-6.99; 2H, d, 6.65-6.64] represented phenyl ring of tyrosine. A doublet signal at [δ, 2H, d, 2.73-2.78; $-CH_2$ of tyrosine] represented $-CH_2$ protons, one triplet at (δ, 4.41-4.43, 1H, t) as one methine proton and one doublet signal at (δ, 2H, d, 2.93-2.98; $-CH_2$ of glycine) indicated $-CH_2$ protons. The free hydroxyl group of carboxylic acid showed a sharp singlet peak at 12.98 (δ 1H, s, -OH). The proton NMR signals clearly confirmed the structure of metal free glycyl-tyrosine compound. After copper metal incorporation into the structure of the glycyl-tyrosine compound (Figure 4), free $-NH_2$ and -OH group signals were disappeared in the proton NMR spectrum due to the coordination with the central metal ion. From mass spectrum, the molecular formula of the copper (II)-glycyl-tyrosine was elucidated based on their molecular ion peak M^+ [299.0177]. Further confirmation of the structure [Cu(II)-glycyl-tyrosine] of the complex was made by ICP-OES spectral analysis as shown in the table 5.

Docking studies have been carried out using Hex software tool. The designed metal incorporated linear peptides were docked with different target cancer receptors collected from the protein database bank, out of all receptors HPV18-2IOI receptor showed good binding interaction with the (ligand) Metallopeptides. The docking results were shown in table 6.

Table 5: ICP-OES spectral data of metallopeptides 5(a-d) & 6(a-d)

Metal complexes	Analytical λmax	ICP Conc. mg/L
Cu(II)-glycyl-glycine (**5a**)	327.393	56.26
Cu(II)-glycyl-tyrosine (**5b**)	327.393	57.19
Cu(II)- alanyl-leucine (**5c**)	327.393	56.82
Cu(II)-alanyl-alanine (**5d**)	327.393	56.97
Ni(II)-glycyl- glycine (**6a**)	231.604	72.09
Ni(II)-glycyl-tyrosine (**6b**)	231.604	73.62
Ni(II)- alanyl-leucine (**6c**)	231.604	73.93
Ni(II)- alanyl-alanine (**6d**)	231.604	72.57

Table 6: Docking results 5(a-d) & 6(a-d):

Synthesized Compounds	PDB Code	E total (KJ/Mol)
Cu(II)-glycyl-glycine (5a)	2IOI	-89.15
Cu(II)-glycyl-tyrosine (5b)	**2IOI**	**-160.11**
Cu(II)- alanyl-leucine (5c)	2IOI	-73.14
Cu(II)-alanyl-alanine (5d)	2IOI	-68.17
Ni(II)-glycyl- glycine (6a)	2IOI	-57.89
Ni(II)-glycyl-tyrosine (6b)	**2IOI**	**-141.65**
Ni(II)- alanyl-leucine (6c)	2IOI	-112.46
Ni(II)- alanyl-alanine (6d)	2IOI	-74.34

Based on the docking results, only two compounds were screened for their *in vitro* anticancer activity against human cervical cancer cell line (HeLa). The samples were prepared at five different concentrations in dimethylsulfoxide (10, 20, 50, 75, 100 µg/mL). 5-fluorouracil is one of the most effective anticancer agents was used as a reference drug in the study. The relationship between surviving fraction and drug concentration were plotted to obtain the survival curve of cervical cancer cell line. The response parameter calculated was the IC_{50} value, which corresponds to the concentration required for 50% inhibition of cell viability. The metal complexes **5(b) & 6(b)** showed a potent anticancer activity profile against HeLa cell lines with IC_{50} values less than 50 µg/mL. The percentage of cell death was given in Table 7.

Table 7: Anticancer activity of synthesized metallopeptides

Anticancer activity of Synthesized Metallopeptide complexes on HeLa cancer cells[a]		
Metallopeptides	**Percentage of viable cells**	
	Control	**Treated**
Cu(II)-glycyl-tyrosine 5(b)	100	36
Ni(II)-glycyl-tyrosine 6(b)	100	29

[a]Values are mean of the three experiments. The viable HeLa cells were calculated after 48 hrs of synthesized metallopeptides treatment and strained with trypan blue dye exclusion test.

CONCLUSION

A new series of metal incorporated dipeptide derivatives were synthesized in good yields. Molecular docking studies conclude; the designed metallopeptides showed greater affinity towards HPV18-2IOI target protein for HeLa cancer. Based on the docking results, the synthesized complexes were screened for their anticancer activities. Tyrosine containing metal complex showed potent anticancer activity against HeLa cancer cells.

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