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ARTICLE

MCR-Click Synthesis, Molecular docking and Cytotoxicity evaluation of a new series of Indole-Triazole-Coumarin hybrid Peptidomimetics

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Rajeena Pathoor and D. Bahulayan*

The design and synthesis of a new series of indole–triazole-coumarin hybrids as potential CDK2 inhibitors is described. The new hybrid molecules were synthesized via copper (I) catalyzed [3+2] azide-alkyne cycloaddition and were showed excellent binding affinity towards CDK2 kinase when subjected to virtual screening based on molecular docking. The molecular docking results were experimentally validated by cytotoxicity evaluation against human breast cancer cell lines MCF-7 and western blot Analysis. The IC50 value (17.5µM) and binding affinity (-11.2 Kcal/mol) obtained for **6a** against MCF-7 cells are promising for the development of potential anticancer drugs based on these new molecules.

Introduction

Cyclin-dependent kinase 2 (CDK2) otherwise known as cell division kinase is an enzyme belonging to Serine/Threonine kinase family which plays a key role in cell cycle regulation such as cell cycle propagation, neuronal function, differentiation and apoptosis.[1, 2] The overexpression of CDK2 will lead to multiple type of malignancies such as colorectal, ovarian, breast, lung, pancreatic and breast cancer etc.[3] Breast cancer is one of the leading cause for the increase in female death rate and recent studies show that CDK2 has a crucial role in human breast cancer, for example the irregular CDK2-AP1 expression can cause the proliferation of breast cancer cell lines.[4] Similarly, the elevated expression of cyclin E in primary tumors has also been shown to be one of the reasons for the poor survival rate in breast cancer patients.[5] Several CDK2 inhibitors have been reported over the years based on privileged scaffolds such as purines,[6] pyridines,[7] pyrimidines,[8] thiazoles,[9] triazoles,[10] imidazoles,[11] indoles [12] etc. and many of them have excellent IC50 up to nanomolar level against various malignant cell lines (see Fig.1 for typical examples[1b,2]). Very recently, FDA has given approval for ribociclib (Kisqali®) and expanded its earlier approval of palbociclib (Ibrance®) for the initial treatment of breast cancer. However, the synthesis of most of these drug molecules involve multistep synthetic protocols with the involvement of large amount of resources infrastructure, manpower etc. leading to the escalated prize of such medicines. Hence, the investigations to develop alternatives to such costly synthesis is essential. Skilful use of step economic synthesis such as multicomponent coupling reactions (MCR)[13] and close to natural fragment assembly methodologies such as click chemistry[14] can contribute greatly to achieve this goal.

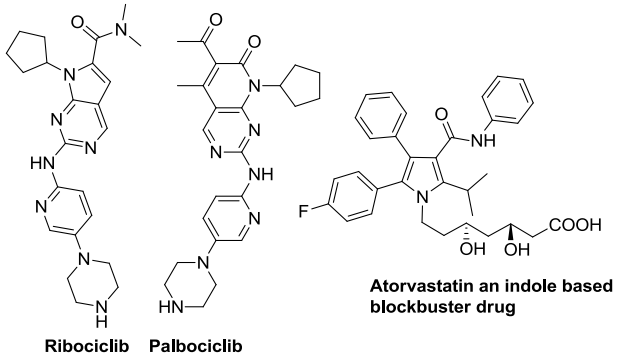

Fig. 1 Typical examples of FDA approved CDK2 inhibitors and a potential example of indole based anti-cholesterol drug.

Inspired by the recent developments in the field of click chemistry and multicomponent reactions for the speedy delivery of functional scaffolds for drug discovery, we decided to undertake the peptidomimetic derivatization of a core indole scaffold with 1,2,3-trizole moieties to obtain triazole linked peptidomimetics with potential biological activity.

Our rationale for choosing indole as a core scaffold for peptidomimetic randomization is due to its large recurrence in bioactive natural products and synthetic drugs. Potential example of a synthetic drug with indole as core unit is also presented in Fig.1.

Experimental section

General

All reactions were carried out with oven-dried glassware. Starting materials were purchased from Aldrich and Merck. High resolution mass spectra were measured with DMSO as

[a] Department of Chemistry, University of Calicut, Malappuram 673635, Kerala, India. E-mail: bahulayan@yahoo.com

† Footnotes relating to the title and/or authors should appear here.
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solvent. IR spectra were recorded on a JASCO-FTIR-4100 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker FT-400 & 100 MHz, using TMS as an internal standard.

General procedure for the synthesis of indole functionalized alkynes 1a-1c.

Indole derivative (1mmol) and potassium carbonate (414.63mg, 3 mmol) were dissolved in minimum amount of DMF, and stirred at 50^{o}C for 10 min. After cooling the reaction mixture to room temperature, propargyl bromide (118.96 mg, 1 mmol) was added to it and stirred for another 4 h and then poured into ice cold water. The solid product obtained was filtered and dried under vacuum to obtain indole functionalized alkynes **1a-1c**.

1-(prop-2-yn-1-yl)-1H indole-3-carbaldehyde (1a)

White solid ; 159 mg (yield 87%); Mp: 90-93^{o}C ; ^{1}H NMR (DMSO-d6 , 400 MHz) δH (ppm): 9.94 (s, 1H); 8.37-8.35(d, J=8Hz, 1H); 8.15-8.12 (m, 1H); 7.66-7.64 (m, 1H); 7.38 - 7.28 (m, 2H); 5.24 (s, 2H); 3.43 (s, 1H); ^{13}C NMR (DMSO-d6,100 MHz) δC (ppm): 185.4 ,140.56, 137.05, 125.17, 24.23, 123.43, 121.78, 118.34 ,111.85, 78.43,77.20, 40.45; IR (KBr) υ max :3198, 2121, 1656, 1644, 1615, 1579, 1528, 1471, 1441, 1308, 1244, 1163, 1041, 770, 741, 562 cm^{-1}; MS (ESI): m/z =184 (M +1).

General procedure for the synthesis of coumarin functionalized azides 3a-3d.

A mixture of corresponding benzaldehyde (1 mmol), 3-acetylcoumarin (188 mg, 1 mmol), and 3-bromopropionitrile (133 mg, 1 mmol) in acetonitrile (8 ml) were stirred in the presence of catalytic amount of $CuSO_4$ at room temperature for 4 h. Subsequently the reaction mixture was poured in to ice cold water and extracted with CH_2Cl_2 (15 mLx2). Evaporation of the solvent followed by purification on a silica gel column (100–200 mesh), by eluting with ethyl acetate/hexane (3:1) afforded the corresponding *β*-amidoketonebromide **2** (see Supplementary Information for spectral data)**. 2** (1 mmol), K_2CO_3 (414 mg, 3 mmol), NaN_3 (65 mg, 1 mmol) were then dissolved in dimethylacetamide and stirred for 6–8 h and then poured into ice cold water. The precipitate was filtered and dried under vacuum to afford the azides **3a**-**3d**.

General procedure for the Cu (I) catalysed 1, 3-dipolar cycloaddition reaction for the formation of indole-triazole-coumarin.

An equimolar amount 1-(prop-2-yn-1-yl)-1H indole-3-carbaldehyde **1a** (91.53 mg, 0.5 mmol) and the coumarin azide **3b** (239.07 mg, 0.5mmol) were dissolved in minimum amount of DMSO. To this, 2 ml of *t*-BuOH, 1 ml of water, $CuSO_4.5H_2O$ (200 mg) and sodium ascorbate (150 mg) were added and stirred at room temperature for 12 h and then poured in to cold water. The precipitated click product was filtered, washed with water and dried under vacuum to afford **4d** in pure form (555.79 mg, 84%).

N-(1-(4-chlorophenyl)-3-oxo-3-(2-oxo-2H-chromen-3yl) propyl)-3-(4-((3-formyl-1H-indol-1-yl) methyl)-1H-1,2,3-triazol-1-yl) propanamide (4b)

Brownish solid ; 522.92 mg (yield 86%);^{1}H NMR (400 MHz, DMSO-d6): δH (ppm): 9.88 (s, 1H), 8.41-8.36 (d, J=20Hz,1H), 8.36-8.34 (d, J=8Hz, 1H), 8.30 -8.27 (d, J=8Hz ,1H), 8.21 (s, 1H), 8.12 (s, 1H), 7.73-7.71 (d, J= 7 Hz, 1H),7.66 (s, 1H), 7.63-7.17 (m, 8H), 6.36-6.341(m, 1H), 6.22 (s, 2H), 5.93 -5.87 (dd, J= 8Hz and J=16Hz , 1H), 5.48-5.43 (dd, J= 8Hz and J= 20Hz, 1H), 5.06-5.01 (m,2H), 1.35 -1.23 (t, 2H); ^{13}C NMR (100 MHz, DMSO-d6) δC (ppm): 199.93, 185.42, 177.79, 166.54, 159.39, 141.11, 139.87, 137.08, 131.96, 129.58, 125.30, 124.30, 124.16, 123.33, 123.26, 123.11, 121.66, 121.58, 121.55, 117.87, 112.58, 111.59, 112.58, 111.66, 111.24, 90.92, 77.21, 48.13, 46.13, 36.50; IR(KBr)υmax: 3309, 2935, 2745, 2676, 2491, 1641, 1580, 1528, 1452, 1393, 1315, 1241, 1196, 1030, 750, 428 cm^{-1}; HRMS m/z ; 608.1699 (calc. 608.1701).

3-(4-((3-acetyl-1H-indol-1-yl) methyl)-1H-1,2,3-triazol-1-yl)-N-(1-(4-chlorophenyl)-3-oxo-3-(2-oxo-2H-chromen-3-yl)propyl)propanamide (5b)

Brownish solid ; 516.32mg (yield 83%); ^{1}H NMR (400 MHz, DMSO-d6): δH (ppm): 8.58 (s, 1H), 8.38 (s, 1H), 8.30 (s, 1H), 8.21 -8.19 (d, J= 6Hz ,1H), 8.21 (s, 1H), 7.97 (s, 1H), 7.62 -7.61 (d, J= 6Hz,1H),7.48 (s, 1H), 7.43 (s, 1H), 7.31 -7.26 (m, 6H), 5.24 (s, 2H), 5.19-5.01 (m,1H), 4.30 -4.19 (m, 2H), 3.31-3.26 (dd, J=8Hz,16 Hz,1H), 3.19-3.01 (dd, J= 10Hz, 1H), 2.46 -2.35 (m, 2H), 1.23 (s, 3H); ^{13}C NMR (100 MHz, DMSO- d6) δC (ppm): 195.13, 185.22, 168.60, 167.48, 160.72, 141.07, 140.56, 139.86, 138.65, 137.31, 137.05, 129.48, 128.98, 125.13, 124.22, 124.15, 124.06, 123.32, 123.27, 123.09, 121.65, 121.57, 121.54, 118.12, 118.04, 112.57, 112.01, 111.61, 111.58, 111.23, 60.72, 51.69, 50.91, 48.10, 28.54, 28.32; IR(KBr)υmax:3218, 2923, 1716, 1680, 1627, 1609, 1525, 1488, 1455, 1388, 1340, 1275, 1207, 1090, 1013, 944, 931,827, 7 53, 681, 657 cm^{-1}; HRMS m/z ; 622.1854 (calc. 622.1857).

(1-(3-((1-(4-chlorophenyl)-3-oxo-3-(2-oxo-2H-chromen-3-yl)propyl)amino)-3-oxopropyl)-1H-1,2,3-triazol-4-yl)methyl 1H –indole-3-carboxylate (6b)

Brownish solid ; 505.47 mg (yield 81 %); ^{1}H NMR (400 MHz, DMSO-d6): δH (ppm): 9.911(s, 1H),8.29 (s,1H),8.24 (s, 1H), 8.13 (s, 1H), 7.99 (s, 2H), 7.76 (s, 2H), 7.76 -7.63 (m, 2H), 7.49-7.04 (m, 6H), 5.40 (s, 2H), 5.32 (t, 1H), 5.06 -4.92 (t, 2H), 4.83-4.71 (dd, J= 20 Hz ,1H), 3.93 -3.87 (dd, J= 10 Hz and 12 Hz, 1H), 1.29 -1.23 (m, 2H); ^{13}C NMR (100MHz, DMSO-d6) δC (ppm): 194.03, 185.17, 168.21, 167.69, 147.81, 141.05, 140.05, 140.65, 140.53,

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139.87, 137.32, 137.04, 125.13, 124.28, 124.23, 124.14, 124.05, 123.30, 123.24 ,123.08, 121.65, 121.57, 121.54 ,112.57, 112.03,111.65,111.62,111.59, 85.32, 60.92, 51.68, 48.12, 28.54; IR (KBr) υmax: 2974, 2932, 2738, 2676 ,2490, 1748, 1655, 1591, 1528, 1456, 1397, 1360,1337, 1314, 1243, 1170, 1243, 1170, 1122, 1089, 1036, 989, 939, 821,754 cm-1; HRMS m/z; 624.1650 (calc. 624.1650).

CDK-2 binding studies of the Indole-Triazole-Coumarin hybrid Peptidomimetics

Molecular docking

Docking studies were carried out using the Auto Dock Vina 1.1.2. The pdb structure of 1GII (protein structure) was retrieved from the Brookhaven protein database (http://www.rcsb.org). Subsequently, the water molecules and the original inhibitors were deleted from the protein structure. The 3D structure of the synthesized compounds were provided using Marvine Sketch 5.8.3, 2012, ChemAxon (http://www.chemaxon.com) and was converted to pdbqt coordinate by Autodock Tools (ADT; version 1.5.4). After removing all water molecules, polar hydrogens were added, and charges were assigned using the Kollman united atom library using Autodock Tools (ADT; version 1.5.4). To assign the docking sites, all maps were calculated with 1 Å spacing between grid points by Auto grid. The center of the grid box was placed at the center of donepezil with coordinates x =3.31, y =5.159, z =30.05 and with exhaustiveness 8. The dimensions of the active site box were seted at 20 X 22 X 25 Å. Then these parameters were noted into conf file split out file using vinasplit and analyzed the docking interaction using autodock tool. (See supplementary information for the detailed procedure).

Cell culture and treatment

The MCF-7 cells were maintained in RPMI medium 1640 supplemented with 10% fetal bovine serum as well as 100 µg/mL streptomycin, 100 U/mL penicillin, 2 mM L-glutamine and Earle's BSS was adjusted to contain 1.5 g/L Na bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM of Na pyruvate in a humidified atmosphere containing 5% CO_2 at 37°C.

Cytotoxicity assay

Cytotoxicity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MCF-7cells in the log phase were seeded in 96-well plates at a concentration of 1.0 X 10^4 cells/well and incubated overnight at 37°C in 5% CO_2 humidified environment. The cells were then treated with different concentrations of the sample **4d**, **5d**, **6a** and **6d** such as 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM/mL (dissolved with RPMI medium1640) respectively. Controls were also cultivated under the same conditions without the addition of compound solution. The treated cells were incubated for 48 h and then subjected to MTT assay. The stock concentration (5 mg/mL) of MTT-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was prepared and 100 µL of MTT was added in each compound solution treated wells and incubated for 4 h. Purple colour formazone crystals were observed and these crystals were dissolved in 100 µL of dimethyl sulphoxide (DMSO), and read at 620 nm in a multi well ELISA plate reader (Thermo, Multiskan).

Cell morphology

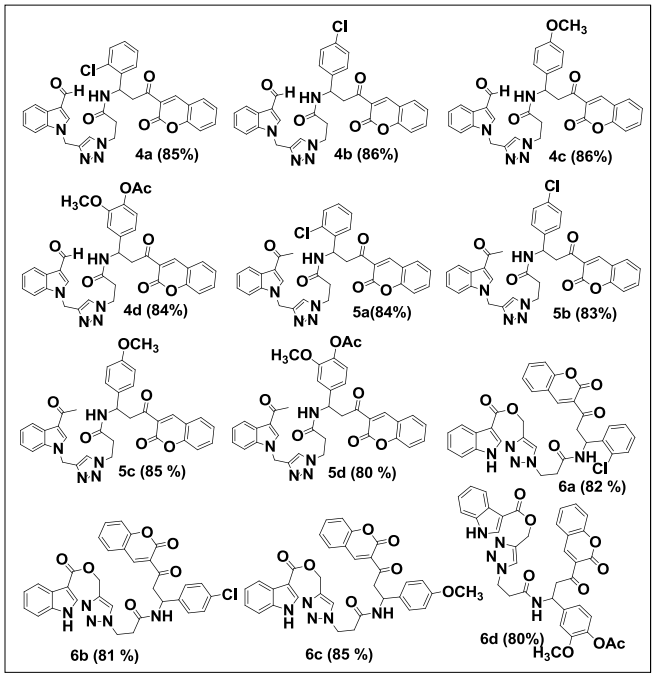
MCF-7 cells were grown (1X10^5cells/cover slip) and incubated at its IC50 concentration and then they were fixed in a mixture of methanol and acetic acid (3:1, v/v). The cover slips were gently mounted on glass slides for morphometric analysis. Morphological changes of MCF-7 cells were analysed under a Nikon (Japan) bright field inverted light microscope at 40 magnification. Followed by this, DAPI (4,6-diamidino-2-phenylindole, dihydrochloride) staining was carried out. For this, the MCF-7 cells incubated with compounds at their IC50 concentrations for 48 h and then they were fixed in a mixture of methanol and acetic acid (3:1, v/v) prior to washing with PBS. The washed cells were then stained with 1 mg/mL DAPI (4,6-diamidino-2-phenylindole, dihydrochloride) for 20 min. in a dark atmosphere. The stained images were recorded using a fluorescent microscope with appropriate excitation filter.

Results and discussion

The concept of multicomponent coupling reactions and click chemistry were effectively utilized for developing the protocol and the overall sequence involved in the synthesis of the indole-triazole-coumarin hybrids is presented in scheme **1**. The studies were started with the synthesis of the alkyne functionalized 3-substituted indole fragment **1** through a base catalyzed condensation reaction between the respective substrates as shown in step **1**. The coumarin azide fragments **3** were synthesized by following a two-step process employing a Mannich type four component reaction to afford the bromo derivative **2** followed by substituting the bromine in **2** with an azide moiety to afford **3.** Those scaffolds substituted with electron withdrawing groups at their phenyl ring gave better yield for the azides compared to those with electron donating groups at the same position. The fragments **1** and **3** were assembled via CuAAC to obtain the peptidomimetic **4** spaced with a linker 1,2,3-triazole. These click reactions were done at room temperature in a mixed solvent system containing *t*-butanol, water and DMSO in the ratio 4:2:1 using $CuSO_4$ as Cu (I) source and sodium ascorbate as the reducing agent. All the click reactions afforded excellent yield for **4a-6d** as shown in table 1 and the structures were characterized via FT-IR, ^{1}H NMR, ^{13}C NMR and HRMS analysis.

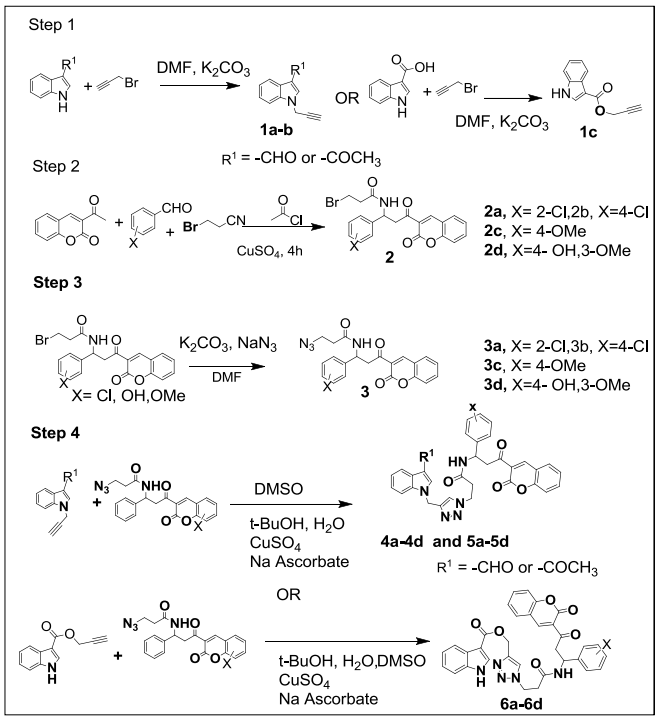
Having synthesized the peptidomimetics, we then moved on to the calculation of the drug property descriptors of these new molecules using molinspiration property calculation service (see supplementary information for descriptor values).[15] The values obtained are slightly higher than that allowed by Lipinski's rule of 5 (Ro5) and are best suited to include in the extended Ro5 space (see SI, Table.S3). Recent analysis of

extended Ro5 class of molecules have revealed that such molecules have significant possibilities as orally bioavailable and cell permeable drug candidates for undruggable targets.[16]



Scheme 1. Overall protocol for the synthesis of indole-triazole-coumarin peptidomimetics.

Table 1. Synthesis of Indole-triazole-coumarin hybrids via CuAAC reaction.



Molecular docking of 4a-6d for studying CDK-2 binding interaction

The promising results obtained from the drug-likeness calculations prompted us to perform the virtual screening of our molecules against CDK 2 based on molecular docking .[17] CDK-2 binders generally interact with CDK-2 proteins via hydrogen bonding, hydrophobic and Pi-cation interactions with various amino acid residues present in their active sites.[18] Protein target with PDB id1GII was downloaded from web source.[19] The active site of CDK2-1GII comprised of amino acid residues such as 31 Alanine, 44 Alanine , 132Aspargine, 86 Aspartic acid, 145 Aspartic acid, 85 Glutamin, 131 Glutamine, 81 Glutamic acid, 84 Histidin,10 Isoleucine,134 Leucine 33 Lysine,80 Phenylalanine, 18 Valine, 64 Valine and 83 Valine.[20] Since most of the amino acid residues in the active site are hydrophobic and hence they are the main contributors for the receptor ligand interaction. PDB id1GII was docked with **4a-6d** using the docking program AutoDockVina 1.1.2 and the binding energies and docking modes were derived. The docking scores obtained are listed in table 2.

From the docking studies we observed that, similar to the parent ligand, the synthesized compounds can also be well embedded in the binding pocket of CDK2 (SI, figure S4). The binding affinity of the parent or inbuilt ligand with CDK2-1GII is -9.5kcal/mol. Compound **4a** sit in the active sit of the receptor protein (no H bonding) with a docking score of -10.9 Kcal/mol whereas compound **4b** interact with CDK-2 by forming two hydrogen bonds with the carbonyl part of the indole and His 82 with a docking score of -9.7Kcal/mol. **4c** does not form any hydrogen bonds with CDK-2 but well embedded in its active site with a binding score of -10.1Kcal/mol. **4d** interact with receptor protein by forming two H-bonds, one between carbonyl group of the indole with HIS 82 and the other one between the acetyl group of the indole with THR14 residue of the α-helical part of the enzyme with a binding affinity -9.8 kcal/mol. **5a** interact with the receptor protein by forming one hydrogen bond with the carbonyl group of indole with HIS 82 with a binding score of -10.6Kcal/mol whereas **5b**, **5c** and **5d** were found to interact with the receptor protein with binding scores -10.8 Kcal/mol, -10.0 Kcal/mol and -10.1Kcal/mol respectively and without forming any hydrogen bonds. This trend was followed in compounds **6a** and **6b** and these molecules were also interacted with CDK-2 with binding scores -11.2 Kcal/mol and -10.3 Kcal/mol respectively and without forming any hydrogen bonds. **6c** and **6d** were also interacted with CDK-2 with binding scores of -9.3 Kcal/mol and -10.0 Kcal/mol respectively. **6c** formed one hydrogen bond with the amide NH of the ligand whereas **6d** formed three hydrogen bonds with a) ester oxygen of indole with HIS82, b) carbonyl oxygen of the coumarin ring with GLU 12 and c) NH group with GLY 13 residue of the protein. Among the 12 compounds studied, **6a** showed the highest binding score of -11.2 Kcal/mol (Fig.2). In addition to this, all the molecules showed a Pi-cation interaction with the receptor and ligand where the coumarin core and other aromatic rings act as Pi system and the NH_3^+ (zwitter ion) of the CDK-2 protein act as the cation.

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Table 2. Docking scores of compounds **4a-6d**

Compound	Docking score / binding affinity (Kcal/mol)	Interactions with receptor
4a	-10.9	ALA 31, PHE 80, ASP145,VAL 18,LYS 20 ALA 144, ASP 86, LEU 1, HIS 84,VAL 64
4b	-9.7	VAL 64, PHE 80, ALA 31, ALA 144, GLY 13, LYS 129, LEU 134, HIS 82,(HB), LYS 20, ASN 132, ILE 10.
4c	-10.1	VAL 64, PHE 80, HIS 82, ASP 145,ALA 144 LYS 20, VAL 18, GLU 12, GLU 8, GLY 11 ILE 10, LEU 134, GLN 85, ASP 86, THR 89
4d	-9.8	THR 14(HB),GLY 13,GLU 12,GLY 11,ILE 10, HIS 82(HB), LEU 134, ALA 31, PHE 80, LYS 120
5a	-10.6	ALA 31, VAL 18, GLU 12, GLY 13, GLY 11 ILE 10, HIS 82(HB), ASN 132, LYS 129, GLN 131, LEU 134
5b	-10.8	PHE 80, VAL 18, GLU 8, LYS 20, ASP145 VAL 64, ALA 144, HIS 82, ILE 10, GLN 131 LEU 134, ASP 86, THR 89
5c	-10.0	LYS 20, ILE 10, ASN 132, LYS 129,GLN 131, LEU 134, GLN 85, ASP 86, LEU 298, LEU 134
5d	-10.1	LYS 120,THR 14, GLY 13, GLU 12, GLY 11, ILE 10,HIS 82, ALA 31,LEU 134, ALA 144
6a	-11.2	PHE 80, ALA 31, LYS 20, HIS 82, VAL 64, GLU 81, GLY 11, GLU 8, ILE 10, VAL 83 LEU 134, HIS 84, ASP 86
6b	-10.3	ALA 31, PHE 80, LYS 20, GLU 8, ILE 10, VAL 64, GLU 81, VAL 83, LEU 134,ASP 86 THR 89
6c	-9.3	VAL 18, ILE 10(HB), LYS 20,ALA 31, ASP 145, HIS 82, VAL 83, PHE 80, LEU 134
6d	-10.0	LYS 120, GLY 13(HB), GLU 12(HB), ILE 10, VAL 18,THR 89, HIS 82(HB), PHE 80, LEU 134, ALA 144, ASP 145

[a]HB= Hydrogen bonding

In order to study the effect of water molecules in the binding interaction of the ligands, the molecules were also docked against the target in presence of water molecules in the crystal structure. However, water molecules did not form any hydrogen bonds with the ligands and also not influenced the binding energy of the molecules we obtained in the absence of the water molecules.

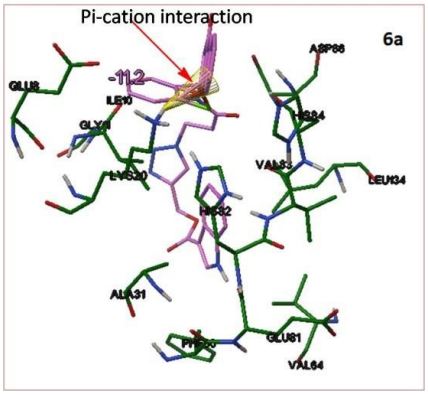

Fig. 2 The binding mode of compound**6a** (high docking score) in the active site of CDK2-1GII

***In vitro* cytotoxic activity against MCF-7 cell lines**

The cytotoxicity of **4d, 5d**, **6a** and **6d** against human breast cancer cell lines (MCF-7) were evaluated using MTT assay (see SI, Fig.S3) for validating the results obtained from molecular docking.[21] The molecules showed IC50 in the range of 17.5-40 µM. The molecules **6a** which showed the highest binding affinity has also showed the lowest IC50 **(**17.5µM) against MCF-7 cell lines followed by **4d** which showed an IC50 of 20 µM against MCF-7 cells. However, we didn't observe any trend in IC50 with respect to the binding energy obtained from computational method.

The presence of strong electronegative or electron donating groups at the core aromatic ring has a strong influence on the lipophilicity of molecules which can alter the cytotoxicity of the drug candidates. In compound **4d**, an electron withdrawing –CHO group is present at the third position of the indole ring and which could be the reason for the enhanced cytotoxicity observed for **4d**. In the case of **5d**, instead of the aldehyde functionality, a keto group is present at the 3-carbon of the indole which is less electron withdrawing compared to the aldehyde group and therefore the compound afforded a slightly high value for the IC50 (30µM). While comparing **6a** and **6d**, both compounds have same substitution at the third position of indole ring, but **6a** showed the least IC50 value (17.5 µM) and **6d** showed the highest IC50 (40µM) . This could be due to the influence of high electron releasing ability of the –OH and –OCH_3 groups in **6d** compared to the electronegative character of chlorine in **6a**.

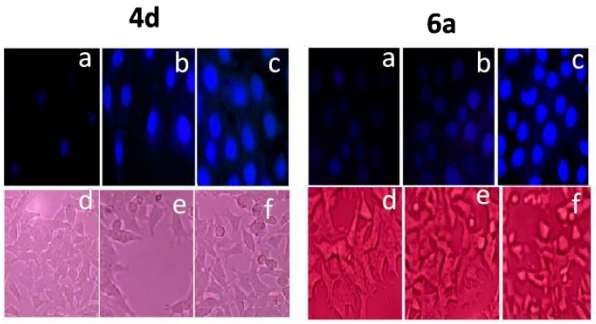

Fig. 3. Fluorescence microscopy images of **4d** and **6a**: (a) control cells, (b)cells treated with **4d or 6a** at their IC25, and (c) cells treated with **4d or 6a** at their IC 50 and bright field inverted light microscopy images of: (a) control cells, (b) cells treated with **4d** or **6a** at IC 25, (c) cells treated with **4d** or **6a** at IC 50.

The morphology of the cells after treatment with **4a**, **5a**, **6a** and **6d** were also studied. For this, the MCF-7 cells were incubated with **4a**, **5a**, **6a** and **6d** at their IC25 and IC50 concentrations and were analysed under a Nikon (Japan) bright field inverted light microscope at 40 magnification followed by DAPI (4,6-diamidino-2-phenylindole, dihydrochloride) staining. The bright filed and fluorescence microscopic images of **4d** and **6a** are also shown in Fig. 3. As shown in Fig. 3 and Fig. S6, the control cells have a normal morphology with intact round nucleus emitting weak fluorescence. However, on treatment with **4d, 5d, 6a** and **6d,** a significant nuclei fragmentation with condensed and apoptotic nuclei was observed and the total number of apoptotic cells were increased with the increase in the

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incubation concertation. It has been reported that, the anti-cancer drug doxorubicin interacts with DNA topoisomerase II (topo II) causing the accumulation of enzyme –DNA leading to the rupture of the double-strand and cell death via apoptosis. [22] Similar patterns of nuclei fragmentations were observed here also when breast cancer cells MCF-7 were treated with **4d, 5d, 6a** and **6d.**

The MTT stained molecules were also observed under a Nikon (Japan) bright field inverted light microscope at 40 magnification. The fluorescence images taken at various concentrations are shown in Fig. 3. As shown in Fig.3 and Fig. S6 (a, b, c), the cells incubated with **4d**, **5d**,**6a** and **6d** at their IC50 concentrations showed maximum fluorescence whereas the control cells without **4d**, **5d**, **6a** and **6d** did not show any detectable fluorescence which indicates the potential of these molecules for developing in bio-imaging agents based on them.

Western Blot Analysis of 6a to study CDK-2 selectivity in MCF-7 cells

Since **6a** afforded highest binding affinity and lowest IC50, we decided to study its CDK2 selectivity via western blot analysis.[23] For this, the MCF-7 cells (1×10^6) were seeded in to 100-mm culture dishes in the presence and absence of **6a** and kept for 48 h. Cells were then washed twice with ice-cold PBS and incubated in lysis buffer. The lysates were centrifuged at 10,000 × g for 5 min (at 4°C) and were used as the cell protein extracts. The extracts were then subjected to 12% SDS polyacrylamide gel electrophoresis after which the proteins were transferred in to a nitrocellulose membrane, and then blocked for 1 h using 10% skim milk in water. After washing in a PBS containing 0.1% tween 20 for 3 times, the primary antibodies were added at a v/v ratio of 1:1000. After overnight incubation at 4 °C, the primary antibodies were washed away and the secondary antibodies were added for 1 h incubation at room temperature. Finally, the enhanced chemiluminescence detection reagents were used to develop the signal of the membrane. As shown in Fig. 4, the protein level of CDK2 are significantly down-regulated in the **6a** treated cells compared to the control when β-actin was used as the internal standard.

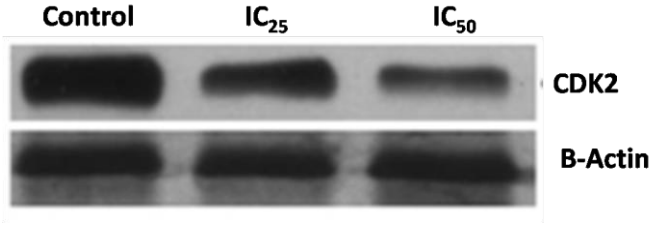

Fig. 4. Western blot Analysis of **6a** against CDK 2 in MCF-7 cell lines

Conclusions

In this paper, we have demonstrated the design and synthesis of twelve indole-triazole-coumarin hybrids as potential inhibitors of protein kinase CDK 2 which is responsible for multiple types of cancers including human breast cancer. The molecules were synthesised via a four step process comprising the synthesis of indole alkynes and small peptide like coumarin azides. The indole alkynes and the coumarin azides were then assembled via copper (I) catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) reaction to afford the indole-triazole-coumarin hybrid peptidomimetics **4a-6d**. The *in vitro* anticancer activity of the molecules has been explored via both theoretically and experimentally. Molecular docking studies with **4a-6d** against CDK2 revealed that, among the twelve molecules, **6a** has the highest binding affinity towards CDK2. In vitro cytotoxicity study with **4a-6d** against human breast cancer cell line MCF-7 were also conducted and here also **6a** showed the lowest IC50 (17.5 μM). The selectivity of **6a** towards CDK2 was further confirmed via western blot analysis using β-actin as standard. The ease in high yield synthesis , ease in library expansion via structure alterations, cost effectiveness and the results obtained from theoretical and experimental anticancer studies are promising to undertake high level computational and chemical/biochemical experiments to to develop cost effective antitumor agents based on these molecules.

Conflicts of interest

There are no conflicts to declare.

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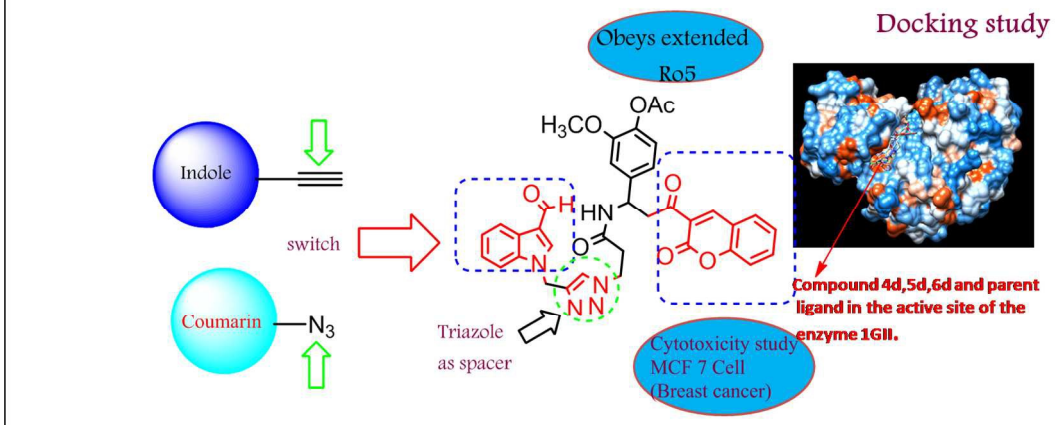
MCR-Click Synthesis, Molecular docking and Cytotoxicity evaluation of a new series of Indole-Triazole-Coumarin hybrid Peptidomimetics

Rajeena Pathoor and D. Bahulayan*

Department of Chemistry, University of Calicut, Malappuram 673635, Kerala, India



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