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Synthesis, Biological Evaluation and Molecular Docking Studies of C-3 Substituted Coumarin Analogs to Explore their Anti-Proliferative Potential

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Abstract

A series of C-3 substituted coumarin analogs were synthesized and evaluated for their *in vitro* cytotoxicity. These analogs showed good ADMET properties and passed Lipinski's filters for drug-likeness. Some of these synthesized compounds, showed potent anti proliferative activity against human cancer cell lines MCF-7, HeLa and SCC-40. 3ACFA and 3ACTA were found active against HeLa and SCC-40 cell lines respectively with GI50 value for 3ACFA against HeLa as 36.34 µg/ml while SCC-40 exhibited a GI50 value of 38.92 µg/ml. 3ACTA analog exhibited the GI50 value of 25.68 µg/ml against SCC-40. The molecular docking was performed with the active site of cyclooxygenase enzyme and the results were well complemented by the experimental data. The possible binding modes of compounds provided a reasonable explanation for the selectivity. These results highlighted that compounds 3ACFA and 3ACTA might be a promising scaffold for cancer therapy.

Keywords: ADMET, Coumarin, Cytotoxicity, Molecular Docking

Introduction

A number of researchers have synthesized and evaluated the biological activities of containing -CO-NH-N=CH- moiety and prove them as significant antitumor agents [1, 2]. On the other hand, furan, thiophene, pyrrole and pyridine scaffolds have been found in numerous cytotoxic agents **(Figure 1)** [3]. Pyridine derivatives display a wide spectrum of biological activities including anticancer, antiviral, and antioxidant etc [4]. Several furan-based derivatives exhibits potent anticancer and other biological activities [5, 6]. Thiophenes are found in many natural products and incorporated in various synthetic compounds and exhibits cytotoxic activity against several cancer cell types, such as leukemia, ovarian, glioma, renal, and lung [7,8]. However, different coumarin Analogs with a number of heterocyclic moieties has been reported to have biological importance as antiproliferative agents [9-11].

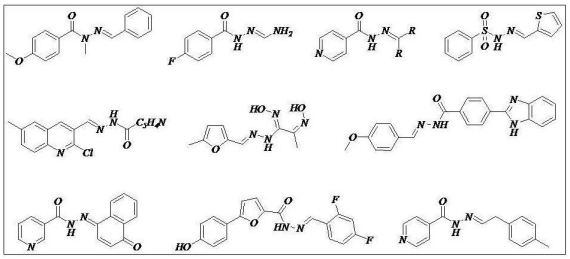


Figure 1. Examples of biological active heterocyclic compounds containing -CO-NH-N=CH- moiety

The coumarin or α-benzopyrone framework is an important pharmacophore, responsible for the various biological activities of coumarins and their derivatives and can interact with various active sites. Hence, coumarin has

been extensively used as a structural subunit for the designing and discovery of various anti-cancer agents [12]. Coumarin is a bioactive framework found in both natural and synthetic sources, prompting biological research to determine their medicinal potential [13]. Coumarin and its derivatives are far and wide distributed in nature and are of great interest with respect to their diverse pharmaceutical activities including anticancer [14], anti-HIV [15], antimicrobial [16], antioxidant [17] and anti-inflammatory [18] activities. Coumarins bearing different substitutions at 3-position are known to exhibit diverse biological activities including anticancer and antitumor activity against different types of cancer cell lines like MCF7, NCIH460, SF268, A549, HCT-116 and HepG-2 **(Figure 2)** [19, 20]. Furthermore, some coumarins with different pharmacophores at C-3 position have been tested for anticancer and anti-inflammatory activities [21, 22]. Thus, in order to develop significant anticancer agents, different aromatic acid hydrazide moieties were introduced to the C-3 position of coumarin frame work and the target compounds, C3-substituted coumarin analogs were designed by using the concept of molecular hybridization which is one of the most important step in drug design [23] to obtain a hybrid with improved pharmacokinetics. It has been reported that the combination of distinct pharmacophores in the same structure is very likely to obtain compounds with significant activity [24].

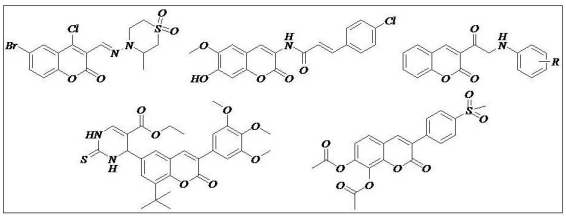


Figure 2. Examples of biological active compounds containing C-3 substituted coumarin moiety

To be effective as a drug, a potent molecule must reach its target in the body in sufficient concentration, and stay there in a bioactive form long enough for the expected biologic events to occur [25]. Computer Aided Drug Design (CADD) methods are useful to identify and develop a potential lead. Similarly, Absorption-Distribution-Metabolism-Excretion (ADME) studies are utilized in the drug development process to examine numerous factors which influence on drug activity and viability [26]. The predicted pharmacokinetic parameters and other physicochemical properties are important for both *in silico* and *in vitro* evaluation of drug-like properties [27]. Hence, in order to obtain new anticancer agents with better activities and higher selectivity, the target compounds were developed by checked for the ADMET and drug-likeness and then synthesized by linking the coumarin and different aromatic acid hydrazides through azomethine group using condensation reaction. The synthesized compounds were evaluated for their *in vitro* anticancer potential against three different human cancer cell lines namely, MCF-7, HeLa, and SCC-40 while the tumour selectivity of compounds were tested on the normal human peripheral blood mononuclear cells (PBMCs). Additional data from *in silico* molecular docking study is incorporated to highlight the binding potential of these anlogs with protein.

Materials and Methods

2.1 Designing of C-3 substituted coumarin analogs

ADMET and pharmacokinetic properties were checked using pkCSM (A Cambridge online source, link: http://biosig.unimelb.edu.au/pkcsm/prediction). The structures of all the synthesized hydrazones and their physicochemical properties were drawn and calculated using Chem Draw 12.0 software. Simultaneously, the SMILE file format of all compounds was obtained from Chem Draw 12.0 to obtain the drug likeness data. pkCSM predictor provides information regarding absorption parameters like Human Intestinal Absorption (HIA), Oral bioavailability, Caco-2 permeability, distribution parameters like Plasma Protein Binding (PPB), Blood Brain Barrier (BBB), metabolism parameters like Cytochrome P450 2D6 (CYP2D6) inhibitor and Cytochrome P450 3A4 (CYP3A4) inhibitor, excretion parameters like renal clearance and toxicity parameters like organ toxicity and genomic toxicities.

Materials

The solvents used were purchased from commercial sources and were further dried by standard protocols. The starting materials such as 3-acetylcoumarin, trifluoroacetic acid and different hetero arylhydrazides were obtained from Alfa Aesar, Mumbai. DMEM and FBS were purchased from Himedia, Mumbai. The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reagent was obtained from G-Biosciences, USA. TLC was monitored using commercially available Aluminium TLC plates coated with silica gel GF254 and the developed plates were visualized by UV light and iodine vapors. Melting points of synthesized compounds were determined with open capillary tube on a VEEGO melting point apparatus. The FTIR and NMR spectroscopic data were obtained from CIF, Savitribai Phule Pune University, Pune and HRMS from NCL, Pune. The *in vitro* anticancer activities were carried out at Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai and ASR Lab, Abeda Inamdar Senior College, Pune.

Syntheses of 3-acetylcoumarin hydrazones

A mixture of 3-acetylcoumarin **(1)** and different aromatic heterocyclic hydrazides **(2)** were dissolved in 1:1 molar ratio in absolute ethanol. Few drops of trifluoro acetic acid were added and the mixture was stirred at room temperature and the reaction was monitored by TLC **(Figure 3)**. After completion of the reaction, the solid obtained was filtered and washed with cold ethanol and recrystallized from ethanol.

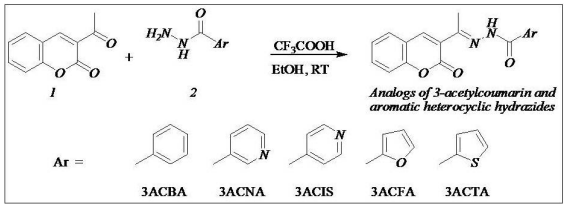


Figure 3. Synthetic scheme for C-3 substituted coumarin analogs

Spectral data of synthesized compounds

2.4.1. (12E)-N'-(1-(2-oxo-2H-chromen-3-yl)ethylidene)benzohydrazide (3ACBA): Off white powder, yield (95%), MP 164-166 ^{O}C; IR (KBr, cm^{-1}): 3186.50 (-NH), 3021 (Ar-H), 1717.87 (Lactone, >C=O), 1658.11 (>C=O), 1604.07 (-C=N-), 1532.80 (Aromatic, C-C);^{1}H-NMR (400 MHz, DMSO) δ 2.32 (s, 3H), 10.83 (s, 1H, -NH), 8.24 (s, 1H, olefinic), 7.88 (d, J = 7.04Hz, 3H),), 7.37 to 7.66 (multiplet, 6H); ^{13}C-NMR (125 MHz, DMSO) δ 164.77(Amide), 159.70(Lactone), 153.88(imine), 142.15, 134.47, 132.91, 132.12, 128.79, 128.58, 127.34, 125.31, 125.08, 124.55, 119.34, 116.56, 16.81(CH3); HRMS (EI) : $C_{18}H_{14}N_2O_3$: 307.10.

(12E)-N'-(1-(2-oxo-2H-chromen-3-yl) ethylidene)nicotinohydrazide (3ACNA)

Yellow, yield (85%), MP 178-180 ^{O}C; IR (KBr, cm^{-1}): 3188.10 (-NH), 3004.12 (Ar-H), 1718.45 (Lactone, >C=O), 1686.55 (>C=O), 1600.68 (-C=N-), 1564.09 (Aromatic, C-C);^{1}H-NMR (400 MHz, DMSO) δ 2.36 (s, 3H),11.08 (s, 1H, -NH), 9.10 (s, 1H, py), 8.81 (s, 1H, olefinic), 8.72 (s, 1H, py), 8.37 (s, 1H, py),8.21 9s,1H, py), 7.95 (s, 1H), 7.74 to 7.40 (m, 3H);^{13}C-NMR (125 MHz, DMSO) δ162.96 (amide), 159.67 (lactone), 155.07 (imine), 153.93, 150.56, 148.14, 138.01, 133.07, 131.24, 129.60, 126.96, 125.29, 124.43, 119.38, 118.63, 116.57, 16.97 (CH_3); HRMS (EI) : $C_{18}H_{14}N_2O_3$: 308.11.

(12E)-N'-(1-(2-oxo-2H-chromen-3-yl) ethylidene)isonicotinohydrazide (3ACIN):

Yellow, yield (88%), MP 190-192^{O}C; IR (KBr, cm^{-1}): 3387.33 (-NH), 3060.67 (Ar-H), 1717.87 (Lactone, >C=O), 1689.30 (>C=O), 1634.28 (-C=N-), 1554.46 (Aromatic, C-C);^{1}H-NMR (400 MHz, DMSO) δ 2.37 (s, 3H),11.13 (s, 1H, -NH), 8.82 (s, 2H, py), 8.29 (s, 1H, olefinic), 7.91 (s, 2H, py), 7.66 (d, 1H), 7.47 to 7.20 (m, 3H);^{13}C-NMR (125 MHz, DMSO) δ 161.55 (amide), 159.66 (lactone), 155.06 (imine), 147.52, 145.66, 134.95, 131.25, 129.95, 126.37, 125.41, 124.94, 124.63, 119.19, 116.62,17.20 (CH_3);HRMS (EI) : $C_{18}H_{14}N_2O_3$: 308.12.

(12E)-N'-(1-(2-oxo-2H-chromen-3-yl) ethylidene)furan-2-carbohydrazide (3ACFA):

Brown, yield (92%), MP 188-190^{O}C; IR (KBr, cm^{-1}): 3178.95 (-NH), 3072.83 (Ar-H), 1705.43 (Lactone, >C=O), 1668.50 (>C=O), 1604.47 (-C=N-), 1561.12 (Aromatic, C-C);^{1}H-NMR (400 MHz, DMSO) δ 2.31 (s, 3H),10.67 (s, 1H, -NH), 8.25 (s, 1H, olefinic), 7.95 (s, 1H, furyl), 7.88 (d, J=7.2Hz, 1H), 7.66 (t, J=7.2Hz, 1H), 7.46 to 7.38 (m, 3H), 6.69 (s, 1H, furyl); ^{13}C-NMR (125 MHz, DMSO) δ 164.74(amide),159.56 (lactone), 153.97 (imine), 146.57, 142.14, 132.95, 129.74, 129.35, 127.12, 125.16, 124.33, 119.30, 116.54, 112.50, 112.38, 16.52 (CH_3); HRMS (EI) : $C_{18}H_{14}N_2O_3$: 297.09.

(12E)-N'-(1-(2-oxo-2H-chromen-3-yl) ethylidene)thiophene-2-carbohydrazide (3ACTA):

White, yield (95%), MP 198-200 ^{O}C; IR (KBr, cm^{-1}): 3164.81 (-NH), 3041.45 (Ar-H), 1723.05 (Lactone, >C=O), 1649.0 1(>C=O), 1615.81 (-C=N-), 1570.85 (Aromatic, C-C); ^{1}H-NMR (400 MHz, DMSO) δ 2.30 (s, 3H),11.10 (s, 1H, -NH), 8.27 (s, 1H, olefinic), 8.05 (s, 1H, thiophenyl), 7.95 (d, J=8.72Hz, 1H), 7.87 (t, J=4.64 & 3.56 Hz, 1H, thiophenyl), 7.67 (t, J=7.4 and 8.08Hz, 1H), 7.40 (m, J=7.4 and 8.24Hz 2H), 7.1 (s, 1H, thiophenyl); ^{13}C-NMR (125 MHz, DMSO) δ 166.30 (amide), 159.65 (lactone), 153.88 (imine), 142.33, 138.15, 135.63, 134.96, 133, 129.78, 129.27, 126.95, 125.31, 124.37, 119.29, 116.56, 16.48 (CH_3);HRMS (EI) : $C_{18}H_{14}N_2O_3$: 313.06.

In vitro anticancer activity (SRB Assay)

The compounds were tested for *in vitro* anticancer activities according to SRB assay protocols available in literature [28]. Cell lines were cultured, inoculated and counted in 96 well plates at four dose levels of test compound concentrations 10, 20, 40, 80 µg/ml. Each experiment was repeated three times. After incubation with different concentrations of test compounds, the cell cultures were stained with SRB dye. The unbound dye was removed by washing with 1% acetic acid and the protein bounded dye was extracted using Tris-HCl buffer base (100 µl, 0.01 M, pH 10.4). The optical density was determined at 540 nm on 96-well plate ELISA reader. The cell viability was expressed as a percentage of the control values.

In vitro cytotoxicity assay against non-cancerous cells

To study the tumour selectivity, the synthesized analogues were tested against non-cancerous normal human peripheral blood mononuclear cells (PBMCs). Isolation of peripheral blood mononuclear cell was done using Ficoll-Hypaque according to the standard method [29] and MTT cytotoxic assay was used to evaluate the cytotoxicity of all the synthesized hydrazones. 1×10^{6} cells per well were seeded in 96-well plate, then exposed to different concentrations of hydrazones (10, 20, 40 and 80 µg/ml) for 24 hours along with a control well. After 24 hours, the culture medium containing hydrazones was removed with one PBS wash. Then 10 µl of MTT solution (5mg/ml) was added and incubated for 4 hours followed by addition of 100µl of DMSO. After 10-20 minutes, absorbance was recorded at 570 nm using Readwell Touch Automatic Elisa Plate Reader (Robonik India Private Limited).

In silico molecular docking

In order to understand the probable binding affinities of the synthesized derivatives, molecular docking studies were carried out at active site of human cyclooxygenase enzyme (COX-2) PDB ID: 6COX using Auto Dock 4.2.614 software. The PDB file of human cyclooxygenase enzyme was downloaded from Royal Society Protein Data Bank (https://www.rcsb.org). The PDB was processed in Discovery Studio for removal of ligand, water molecules and to make the binding sites free for interaction with the synthesized compounds. The processed PBD file was converted to PDBQT file by adding Kollmann charges and further used for docking studies. The images were created with the help of Pymol Molecular Viewer [30].

Results and Discussion

Designing of C-3 Substituted Coumarin Analogs

Online screening of proposed structures for ADME parameters on pkCSM (A Cambridge online source, link: http://biosig.unimelb.edu.au/pkcsm/prediction) showed promising results. pkCSM predictor provides information regarding absorption parameters like Human Intestinal Absorption (HIA), Oral bioavailability, Caco-2 permeability, distribution parameters like Plasma Protein Binding (PPB), Blood Brain Barrier (BBB), metabolism parameters like Cytochrome P450 2D6 (CYP2D6) and Cytochrome P450 3A4 (CYP3A4) inhibition, renal clearance and toxicity parameters like organ toxicity and genomic toxicities. To make sure that the designed molecules show potential as drugs, their ADMET properties were checked and are presented in **Table 1**.

The molecules have a great solubility potential in water. Caco-2 permeability, the human intestinal absorption, skin permeability and CNS permeability are in the permitted range. All

Table 1: ADMET properties of C-3substituted coumarin analogs calculated from pkCSM online tool

Property	Model Name	3ACBA	3ACNA	3ACIN	3ACFA	3ACTA
Absorption	Water solubility[a]	-3.955	-3.432	-3.467	-3.282	-3.978
	$Caco_2$ permeability[b]	0.938	1.098	1.091	1.009	0.908
	Intestinal absorption (human)[c]	93.534	95.88	95.88	94.716	92.01
	Skin Permeability[d]	-2.801	-2.835	-2.845	-2.774	-2.808
Distribution	VDss (human)[e]	-0.343	-0.454	-0.46	-0.415	-0.401
	Fraction unbound (human)[f]	0.026	0.152	0.158	0.189	0.041
	CNS permeability[g]	-1.912	-2.231	-2.218	-2.159	-1.959
Metabolism	CYP2D6 substrate[h]	No	No	No	No	No
	CYP3A4 substrate[h]	Yes	Yes	Yes	Yes	Yes
Excretion	Total Clearance[i]	0.843	0.852	0.887	0.933	-0.002
Toxicity	Max. tolerated dose (human)[j]	0.215	-0.214	-0.242	-0.339	0.115
	Oral Rat Acute Toxicity[k]	2.042	2.15	2.095	2.191	2.104
	Oral Rat Chronic Toxicity[l]	1.214	1.205	1.192	1.12	1.188
	Hepatotoxicity[h]	No	No	No	No	No
	Skin Sensitisation[h]	No	No	No	No	No

[a](log mol/L), [b](log Papp in 10^{-6} cm/s), [c](% Absorbed), [d](log Kp), [e](log L/kg), [f](Fu), [g](log PS), [h](Yes/No), [i](log ml/min/kg), [j](log mg/kg/day), [k](LD50 in mol/kg), [l](LOAEL in log mg/kg_bw/day)

Analogs show the ability to inhibit CYP3A4 and they would not allow the metabolism of xenobiotics in the body. The rate of drug elimination divided by its plasma concentration which is expressed as total clearance is quite favourable for all molecules which implies that the molecules would not accumulate in the body and hence are nontoxic. The analogs didn't show hepatotoxicity and skin sensitization. The pharmacokinetic parameters are predicted through the online source SwissADME (http://www.swissadme.ch/) and it appears that the molecules show good drug likeness properties and could be good drug candidates for further studies. They have Drug Likeness (**Table 2**) with zero violations as per the standards defined by Lipinski, Ghose, Veber, Egan and Muegge. The medicinal chemistry parameters for these compounds underline their potential with lead like properties with no violations and they fall under

the category of PAINS (Pan Assay Interference compoundS). This means these molecules can be the starting point in the pursuit of biologically active compounds. These results depict that the compounds have got good ADMET values and have drug-likeness properties.

Structural analysis

Table 2: Drug Likeness of C-3 substituted coumarin analogs calculated from SwissADME online tool

Compound	3ACBA	3ACNA	3ACIN	3ACFA	3ACTA
Mol.Wt.	306.32	307.30	307.30	296.28	312.35
ClogP	2.97	2.94	2.34	2.54	2.39
No. of H-bond acceptors	4	5	5	5	4
No. of H-bond donors	1	1	1	1	1
PSA	71.67	84.56	84.56	84.81	99.91
Lipinski No. of violations	0	0	0	0	0
Ghose No. of violations	0	0	0	0	0
Veber No. of violations	0	0	0	0	0
Egan No. of violations	0	0	0	0	0
Muegge No. of violations	0	0	0	0	0
Bioavailability Score	0.55	0.55	0.55	0.55	0.55
PAINS No. of Alerts	0	0	0	0	0
Brenk No. of Alerts	2	2	2	2	2
Lead likeness No. of Violations	0	0	0	0	0

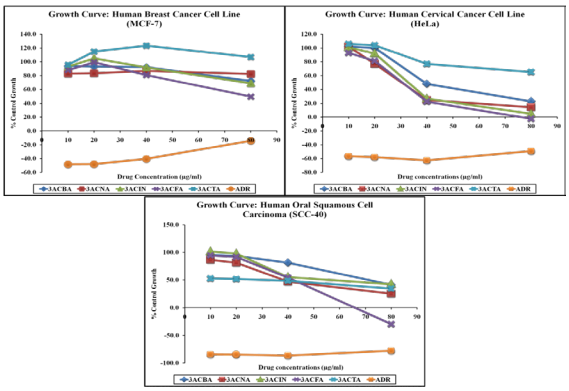
The C-3 substituted coumarin analogs were synthesized by condensation reaction of 3-acetylcoumarin with appropriate aromatic hydrazide in the presence of trifluoroacetic acid under stirring condition at room temperature in absolute ethanol. The structures of the target compounds, **3ACBA, 3ACNA, 3ACIN, 3ACFA** and **3ACTA** were confirmed by mass and spectral analyses. The HRMS (EI) spectra of the synthesized analogs showed major peaks corresponding to expected M+1 fragment at 307.10, 308.11, 308.12, 297.09 and 313.06 respectively. The IR spectra of these analogs showed characteristic peaks for -C=N-, -NH, >C=O (amide) and >C=O (lactone). The IR spectrum of all the analogs showed one peak in between 3164 to 3188 cm^{-1} corresponding to –NH. The peak at 1705 to 1723 cm^{-1} is due to lactone group while the peak at 1600 to 1634 cm^{-1} represents –C=N- group. The ^{1}H-NMR spectrum of the synthesized analogs showed $–CH_3$ protons at 2.3 ppm. The olefin proton on C4 of coumarin ring appeared as a sharp singlet at 8.2 ppm. The aromatic hydrogen atoms were located in the range of 6.6 to 9.1 ppm. The downfield values of aromatic hydrogen atoms were due to the presence of heterocyclic moieties like pyridine, furan and thiophene in the structures. On the other hand, the –NH protons appeared as singlet in the range of 10.67 to 11.13 ppm. The ^{13}C-NMR of all these analogs exhibited signals from aromatic carbon atoms at 112.26 to 151.24 ppm and characteristic peaks of amide, lactone and imine carbons at about 161 to 166, 159 and 153 to 155 ppm respectively. In all these compounds the aliphatic carbon i.e. $–CH_3$ signals were observed in the range of 16.20 to 17.20 ppm.

Anti-cancer activity

The synthesized compounds were evaluated for their *in vitro* anticancer potential against three different human cancer cell lines namely, MCF-7, HeLa and SCC-40 by SRB assay method while the tumour selectivity of compounds were tested on the normal human peripheral blood mononuclear cells (PBMCs) by

MTT Assay. The results for each analogue are expressed as the growth percent of treated cells compared to untreated control cells. The growth curves of the cell lines are shown in **Figure 4**.

Figure 4. Effect of C-3 substituted coumarin analogs on MCF-7, HeLa and SCC-40 cancer cell line



The calculated values of GI50 for test compounds and standard drug Adriamycin (ADR) are shown in **Table 3**. In terms of GI50 values, all the derivatives showed prominent activity against Human cervical cancer cell line (HeLa). **3ACFA** is most active analogue against HeLa and **3ACTA**, against SCC-40 cell lines. The GI50 value for **3ACFA** against HeLa is 36.34µg/m whereas against SCC-40 it is 38.92. **3ACTA** analogue exhibits GI50 value of 25.68µg/ml against SCC-40 while these values of all analogs are more than 80 µg/ml against MCF-7 which are far more than the highest concentration of the drug. When we compare these GI50 values with the standard drug, Adriamycin (ADR) having GI50 value < 10 µg/ml, the synthesized C-3 substituted analogs appear moderately active against all three cancer cell lines.

The results of anti-cancer screening data reveal that the analogs show substantially good activity against HeLa and SCC-40, and are fairly active against MCF-7.

To evaluate cytotoxicity of synthesized Analogs, PBMCs were exposed to synthesized coumarin analogs for about 24 hours. The results are shown in **Table 4**. The hydrazones were non-toxic towards PBMCs at concentration of 10 µg/ml and cytotoxicity increased with increase in concentration. On treatment with 80 µg/ml concentration of each analogue, the percentage growth of cancer cells decreased by 80% in all the cell lines but for PBMCs it decreased by 30% indicating that the synthesized C-3 coumarin analogs are selective against cancer cells.

Table 3: Calculated GI50 (µg/ml) values of synthesized Analogs

Cell Lines	3ACBA	3ACNA	3ACIN	3ACFA	3ACTA	ADR
MCF-7	>80	>80	>80	>80	>80	<10
HeLa	52.48	41.07	41.66	36.34	>80	<10
SCC-40	72.16	48.58	64.67	38.92	25.68	<10

Molecular docking

Table 4: Cytotoxicity studies of C3 substituted coumarin analogs

Codes	3ACBA	3ACNA	3ACIN	3ACFA	3ACTA
Drug concentrations (µg/ml)	% Cell Growth				
10	99.92±2.49	97.75±4.07	99.28±1.70	99.55±1.23	99.37±1.05
20	95.20±3.31	91.84±5.78	95.67±2.62	94.29±1.14	92.72±2.96
40	86.29±2.06	80.02±1.48	87.70±0.46	83.86±2.46	81.32±3.72
80	75.69±1.96	73.51±2.56	76.55±5.13	72.58±2.45	74.89±3.91
Control	100±2.5				

The COX pathway has been linked to the development of different cancer by eliciting growth-related signals and regulating cell proliferation. Under normal conditions COX is expressed at very low levels however gets over-expressed during the inflammatory process, pathogenic stimuli and cancer progression [31]. There are many reports available in the literature suggesting that the tumors can be targeted selectively through COX pathway [32]. In order to understand the plausible binding affinities, we have conducted a molecular docking of the synthesized analogs in the active site of human cyclooxygenase enzyme (COX-2) PDB ID: 6COX was downloaded from Royal Society Protein Data Bank (https://www.rcsb.org) using Auto Dock 4.2.614 software [33]. The estimated binding energy values and the interacting amino acid residues are given in **Table 5**. The obtained values of binding energies reveal that all C-3 substituted coumarin analogs fit satisfactorily into the cyclooxygenase active site of 6COX displaying hydrogen bonding with different amino acid residues of the target protein.

Table 5: Molecular Docking results of C-3 substituted coumarin analogs

Code	3ACBA	3ACNA	3ACIN	3ACFA	3ACTA
B.E. in kcal/mole	-9.5	-9.2	-9.2	-9.9	-9.6
Binding amino acid Residue	ASN-39	ASN-34	ASN-34, GLN-461	CYS-47	ASN-39
Bond Length in Å	3.2	2.5	2.4, 2.5	2.3	2.9

The docking ribbon structures of 6COX protein with respective compounds are shown in **Figure 5**. The best binding energy was exhibited by **3ACFA** followed by **3ACTA, 3ACBA, 3ACNA and 3ACIN**. Thus, the compound **3ACFA** has stronger binding interactions with 6COX than other derivatives. **3ACFA** and **3ACTA** show hydrogen bonding interactions with CYS-47 and ASN-39 amino acid residues and the hydrogen bond distances of 2.3and 2.9 Å indicating strong protein-enzyme interactions which lead to stabilization of compounds in the protein cavity. Based on these results, compounds **3ACFA** and **3ACTA** have better stability in the COX-2 protein cavity than other analogs and anticipated to show more enhanced anticancer activities.

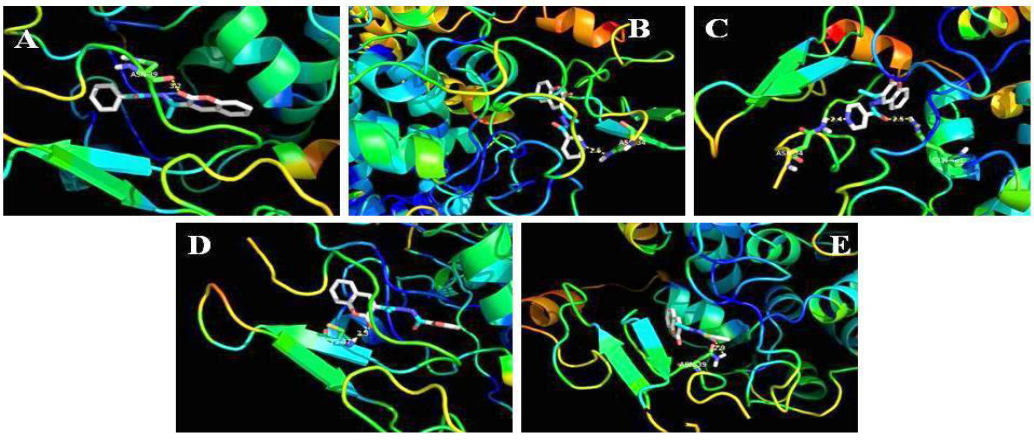


Figure 5. Binding of 3-acetylcoumarin and aromatic heterocyclic hydrazide analogs into active site of 6COX (A = 3ACBA, B = 3ACNA, C = 3ACIN, D = 3ACFA, E = 3ACTA)

Conclusions

ADMET and pharmacokinetic studies were used as support to design a new series of C-3 substituted coumarin analogs as new anticancer agents containing an azomethine. We synthesized C-3 substituted coumarin analogs and the structures of these analogs were confirmed from spectral characterization. The *in vitro* anticancer activities of these analogs were tested against MCF-7, HeLa and SCC-40 and normal human PBMCs. The compounds showed significant activity against HeLa and SCC-40 cell lines. The molecular docking studies are also in best agreement with experimental results. The experimental results can be accounted due to polar interactions between the ligands and amino acid residues in the protein. These compounds fit well in the target active site pocket and the interactions lead to stabilization of the compounds. The analogs possess good ADMET properties and passed Lipinski's filters for drug-likeness. The synthesized analogs exhibit good anti-proliferative activities against HeLa and SCC-40 cell lines which bring in the possibilities of optimizing application of these analogs for future research.

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Conflict of interest

Authors have no conflicts to declare.

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