Synthesis of 3-(2-(substituted-(trifluoromethyl)phenylamino)acetyl)-2H-chromen-2-one derivatives as new anticancer agents

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ABSTRACT

Under solvent free conditions and in presence of a base 3-(2-(substituted-(trifluoromethyl)phenylamino)acetyl)-2H-chromen-2-one derivatives were synthesized by grinding technique. Structural investigations were carried out with IR studies, HRMS, 1HNMR and 13CNMR. The compounds were checked for their in vitro anticancer activities against three different human cancer cell lines viz human breast cancer cell line (MCF-7), human cervical cancer cell line (HeLa) and human oral squamous cell carcinoma (SCC-40) using SRB method. All the title compounds showed low toxicity towards non-malignant PBMC cells indicating their tumour selectivity. The compounds exhibited good in vitro anti-proliferative potency at lower concentrations against HeLa and MCF-7 cell lines and remain moderately active against SCC-40.

Keywords:
Coumarin
Anticancer
MCF-7
HeLa
SCC-40
SRB Assay

1. Introduction

Anti-cancer drugs are meant to target abnormally dividing cell through inhibition of cell division.1 antiproliferative activity of anti-cancer compounds involve diverse mechanism and accordingly such compounds are called as DNA intercalating agents (e.g. adriamycin), DNA cross-linking agents (e.g. cis-platin), topoisomerase inhibitors (e.g. camptothecins), cytoskeleton-disrupting agents (e.g. vinblastin), tyrosine kinase inhibitors (imatinib) and antimitabolites (e.g. mercaptopurine). One of the biggest challenges in chemotherapy is the collateral damage to the normal cells by chemotherapeutic agents and consequent severe side effects and selective activity against cancer cell is desirable attribute in all anti-cancer compounds.2

Coumarins are heterocyclic compounds from family of lactones with 1-benzopyran-2-one system and they can be isolated from essential oils, green tea, fruits and they can be synthesized in the laboratory.3,4 Coumarin is a versatile phytochemical and its derivatives are known to exhibit antibacterial and antimicrobial activities with low toxicity.5,6,7 Coumarin derivatives have also been reported for anticoagulant and anti-inflammatory,8 anti-HIV,9 antioxidant,10 antiallergic,11 anticancer,12 antiproliferative13 and antiviral14 activities and these pharmacological properties depend substituent.15 Coumarins could exert their anticancer activity by different mechanisms either by inhibiting the telomerase enzyme16 and down regulating oncogene expression17 or by inducing the caspase-9 mediated apoptosis. Additionally, researchers showed that coumarins are able to suppress cancer cell proliferation by arresting cell cycle in G0/G1,18 G2/M
phases\textsuperscript{19} and through affecting the p-glycoproteins of the cancer cells.\textsuperscript{20} It was also reported that hydroxycoumarins might exert their anticancer activity by generating free radical species causing oxidative stress leading to pro-apoptotic effects.\textsuperscript{21} It was proven that the \(\gamma\)-lactone ring of the coumarinic system has a fundamental role in both the generation and stabilization of such species as well as in the pro-apoptotic action of hydroxycoumarins.\textsuperscript{22} Moreover, the antiproliferative activity of 7-hydroxycoumarin derivatives could be due to their effect on the mitochondrial thiol.\textsuperscript{23} Many natural and synthetic coumarin compounds (\textbf{Scheme 1}) have shown promising potentials to be anticancer agents. Esculetin (6,7-dihydroxycoumarin, 1) and Scopoletin (6-methoxy-7-hydrocoumarin, 2) which are, typical naturally occurring coumarins displayed antiproliferative effects in human leukaemic cells.\textsuperscript{24} Compound 1 is known to inhibit proliferation and induce cell death in many forms of human cancer cells and is considered as a promising chemotherapeutic agent. Recently, Cho \textit{et al.} reported antiproliferative effect of 2 on the growth of oral squamous cell carcinoma cell lines.\textsuperscript{25} Zhang \textit{et al.} reported potent cytotoxicity of compound 3 against human hepatocellular carcinoma cells.\textsuperscript{26} Chen \textit{et al.} reported compound 4 with remarkable anti-proliferative activity against human prostate cancer and human lung cancer cell line.\textsuperscript{27} Coumarin derivative 5 synthesized by Nasr \textit{et al.},\textsuperscript{28} was found to possess powerful growth inhibitory activity against human hepatocellular carcinoma and human pancreatic carcinoma cell lines. In addition coumarin-containing molecules have been shown to reverse the multidrug resistance (MDR) in various\textsuperscript{29} which underlines the future prospect of coumarin compounds against resistant cancer. The fluorine-containing organic compounds are particularly appreciated in pharmaceutical, agricultural and materials sciences. In recent years, the trifluoromethyl group has attracted more attention, and many trifluoromethylated compounds have been found to possess special activities.\textsuperscript{30} Due to its stereoelectronic property and lipophilicity,\textsuperscript{31} the introduction of trifluoromethyl groups into bioactive molecules has led to the synthesis of new molecules with remarkable therapeutic potential,\textsuperscript{32} for example Celecoxib 6 and SC-558 7 exhibited potent chemo preventive activity as COX-2 inhibitors.

\begin{center}
\textbf{Scheme 1.} Reported Coumarin Derivatives
\end{center}

The sulforhodamine B (SRB) assay is employed for the determination of cell density which is based on the measurement of cellular protein content and the method relies on the stoichiometric binding of SRB with proteins under slightly acidic conditions.\textsuperscript{33-34} Further, molecular docking is a well-known tool to explore binding interaction between drug and receptor. The interaction of a synthesized drug molecules with Cyclooxygenase (COX) target via docking and their relative stabilities have been evaluated using the binding affinities.\textsuperscript{35} Cyclooxygenase (COX) enzymes, also known as prostaglandin-endoperoxide synthase (PTGS), catalyze the metabolic conversion of arachidonic acid (AA) to prostaglandins (PGs) that play an important role in inflammation.\textsuperscript{36} COX-2 is a membrane-bound, short-living, rate-limiting enzyme is a known target for the treatment of inflammation. COX-2 expression is negligible in normal cells but it is expressed frequently at the tumorigenic nests in many types of cancers including adenocarcinoma, squamous cell carcinoma (SCC) and hepatocellular carcinoma.\textsuperscript{37} Recently, benzopyran derivatives were investigated as potent COX-2 inhibitors and more specifically, coumarin derivatives, as a class of benzopyrans were proved to possess potent anti-inflammatory effects and thus were evaluated as COX-2 inhibitors.\textsuperscript{38} Our interest in the identification of new COX-2 inhibitors prompted us to explore the use of coumarin framework for the design of this type of inhibitors.\textsuperscript{38} Researchers have condensed 3-(2-bromo acetyl) coumarin with aniline in ethanol at reflux temperature for 15–30 min which resulted in the formation of 3-(2-(phenylanilino)acetyl)-2H-chromen-2-one but the yield of the products is around 70–75\%.\textsuperscript{39} Though, these methodologies are quite simple and useful but they need prolonged reaction time and give moderate yields of the products. Hence, there is a requirement of alternative procedures with mild reaction conditions and better yields. In the present work we report the synthesis of 3-(2-phenylaminoo)acetyl-2H-chromen-2-ones under solvent-free conditions. The synthesized compounds were tested for their anticancer potential against three cancer cell lines such as human breast cancer cell line (MCF-7), human cervical cancer cell line (HeLa) and human oral squamous cell carcinoma (SCC-40) by SRB method.
2. Results and Discussion

2.1 Spectral Characterization

The HRMS (EI) spectra of 3ACOT, 3ACMT, 3ACPT and 3ACDT showed major peaks corresponding to expected M+1 fragment at 348.0837, 348.0838, 348.0842 and 438.0535 respectively. The IR spectra of these analogs showed characteristic peaks for lactone carbonyl, -NH and >C=O (amide). The IR spectrum of all the analogs showed one peak in between 3020 to 3047 cm⁻¹ for –NH. The peak at 1714 to 1724 cm⁻¹ is due to >C=O (lactone moiety) and that at 1675 to 1686 cm⁻¹ is due to >C=O (ketonic moiety). The ¹H-NMR spectrum of the synthesized analogs showed –CH₂ protons at 4.7 ppm. The olefin proton on C4 of coumarin ring appeared as a sharp singlet at 8.6 ppm. The aromatic hydrogen atoms were located in the range of 6.7 to 7.7 ppm. On the other hand, the protons of –NH groups appeared as singlet at 2.17 ppm. The ¹³C-NMR of all these analogs exhibited signals from aromatic carbon atoms at 109.3 to 149.6 ppm and characteristic peaks for lactone carbonyl, -NH and >C=O (amide). The IR spectrum of all the analogs showed one peak in 1714 to 1724 cm⁻¹ for –NH. The peak at 1714 to 1724 cm⁻¹ is due to >C=O (lactone moiety) and that at 1675 to 1686 cm⁻¹ is due to >C=O (ketonic moiety).

i) 3-(2-(trifluoromethyl)phenylamino)acetyl)-2H-chromen-2-one (3ACOT): Pale yellow powder, yield 90 %, MP 205-206 °C, IR(cm⁻¹): 3020 (NH), 1724 (C=O of lactone), 1685 (C=O of ketone); ¹HNMR (500 MHz, CDCl₃): δ 2.170 (s, 1H), 4.751 (s, 2H), 7.366 to 7.722 (m, 8H), 8.635 (s, 1H, =CH); ¹³C NMR (500MHz, CDCl₃): 35, 116.9, 118.1, 122, 122.1, 122.2, 122.1, 122.1, 125.3, 125.3, 125.3, 125.3, 130.4, 130.4, 135.1, 147.3, 149.6, 158.9, 192.7; HRMS (EI): C₁₈H₁₂F₃NO₃Na: 438.0837

ii) 3-(2-(trifluoromethyl)phenylamino)acetyl)-2H-chromen-2-one (3ACMT): Yellow powder, yield 92 %, MP 198-200 °C, IR(cm⁻¹): 3047 (NH), 1714 (C=O of lactone), 1675 (C=O of ketone); ¹HNMR (500 MHz, CDCl₃): δ 2.166 (s, 1H, -NH), 4.746 (s, 2H, -CH₂), 6.803 (m, 1H), 6.984 (dd, 1H), 6.898 (dd, 1H), 6.85 (dd, 1H), 7.047 to 7.283 (m, 4H), 8.638 (s, 1H, =CH); ¹³C NMR (500MHz, CDCl₃): 54, 110.3, 114.8, 116.8, 121.9, 123.3, 124.6, 125.2, 125.3, 125.3, 125.3, 125.3, 130.4, 135, 149.1, 155.4, 185.8, 188.8; HRMS (EI) : C₁₈H₁₁F₆NO₃: 438.0842

iii) 3-(2-(3-(trifluoromethyl)phenylamino)acetyl)-2H-chromen-2-one (3ACPT): Yellow powder, yield 99 %, MP 210-212 °C, IR(cm⁻¹): 3037 (NH), 1724 (C=O of lactone), 1675 (C=O of ketone); ¹HNMR (500 MHz, CDCl₃): δ 2.166 (s, 1H, -NH), 4.747 (s, 2H, -CH₂), 7.063 (s, 2H), 7.195 (d, 1H), 7.367 to 7.723 (m, 4H), 8.636 (s, 1H, =CH); ¹³C NMR (500MHz, CDCl₃): 53.7, 112.3, 116.9, 121.9, 122.1, 124.1, 125.3, 126.7, 127.2, 127.2, 130.4, 133, 135.3, 149.6, 158.9, 192.7; HRMS (EI): C₁₈H₁₁F₆NO₃Na: 438.0535

iv) 3-(2-(3,5-bis(trifluoromethyl)phenylamino)acetyl)-2H-chromen-2-one (3ACDT): Pale yellow powder, yield 90 %, MP 204-206 °C, IR(cm⁻¹): 3020 (NH), 1724 (C=O of lactone), 1685 (C=O of ketone); ¹HNMR (500 MHz, CDCl₃): δ 2.171 (s, 1H, -NH), 4.751 (s, 2H, -CH₂), 7.366 to 7.722 (m, 8H), 8.635 (s, 1H, =CH); ¹³C NMR (500MHz, CDCl₃): 53.8, 112.2, 119.3, 121.8, 122.1, 124.1, 125.3, 126.7, 127.2, 127.2, 130.4, 133, 135.3, 149.6, 158.9, 192.7; HRMS (EI): C₁₈H₁₁F₆NO₃Na: 348.0837

2.2 Anti-cancer Activity

The anti-proliferative activities of four synthesized coumarin derivatives were tested against human breast cancer cell line (MCF-7), human cervical cancer cell line (HeLa) and human oral squamous cell carcinoma (SCC–40) and the results are depicted in Table 1. The results for are each compound are expressed as the percent growth (GP %) at different concentrations of drug compounds and expressed in μg/ml. Adriamycin (ADR) is used as standard (GP %) to show the standard growth. The curves of different cancer cell lines and normal human peripheral blood mononuclear cells (PBMC) are shown in Fig. 1 to Fig. 4. The activities of test compounds were compared with those of non-malignant normal human peripheral blood mononuclear cells (PBMCs). The results reveal that all the compounds show weak cytotoxicities against normal cells PBMC and thus the findings comply that all compounds were selective against cancer cells.

Table 1. Average values of percentage control growth of 3 cell lines and normal human peripheral blood mononuclear cells (PBMC) at different concentrations in μg/ml

| Cell Lines | Drug Concentrations (μg/ml) | MCF-7 | HeLa | SCC–40 | PBMC |
|-----------|-------------------------|-------|------|--------|------|
|           | 10  | 20   | 40   | 80   | 10  | 20   | 40   | 80   | 10  | 20   | 40   | 80   |
| 3ACOT     | 101.3 | 97.2 | 104.1 | 76.6 | 47.2 | 45.4 | 32.3 | 16.0 | 85.4 | 87.8 | 63.0 | 24.2 | 99.67 | 93.43 | 90.53 | 88.17 |
| 3ACMT     | 97.7 | 103.4 | 97.9 | 76.4 | 48.8 | 49.1 | 26.8 | -12.5 | 98.1 | 95.8 | 78.9 | 27.6 | 97.22 | 93.95 | 87.62 | 80.17 |
| 3ACPT     | 95.5 | 89.5 | 85.0 | 86.2 | 73.9 | 73.8 | 42.2 | 20.9 | 93.3 | 83.4 | 66.0 | 37.7 | 95.29 | 89.27 | 83.72 | 79.98 |
| 3ACDT     | 92.1 | 78.3 | 48.8 | 11.1 | 101.5 | 101.4 | 60.8 | 47.1 | 86.4 | 72.1 | -1.6 | -63.1 | 97.44 | 90.25 | 85.04 | 79.78 |
| ADR       | -48.5 | -48.1 | -40.6 | -14.2 | -56.9 | -58.1 | -62.9 | -49.4 | -84.6 | -84.8 | -86.7 | -77.9 | - | - | - | - |
From the observed data it is noted that all the synthesized compounds show appreciable activity against human cervical cancer cell line (HeLa) compared to other two cell lines. From the anti-proliferative screening data, it is evident that at higher concentrations, the compounds show moderate activities.

Out of the four synthesized derivatives, 3ACDT was found to be most effective against MCF-7 cell lines at 10 μg/ml concentration. 3ACOT and 3ACMT expresses best activity against HeLa cell line at 10 μg/ml concentration. 3ACOH and 3ACMT shows the average cell growth percents of 47.2 and 48.8 respectively against HeLa cancer lines. However, amongst the synthesized coumarin derivatives 3ACOT and 3ACDT were the most active with the average cell growth percents of 85.4 and 86.4 respectively against SCC-40 cell lines. Three compounds show significant anti-proliferative activity against human cervical cancer cell line (HeLa) at lower concentrations in between 47.2 to 73.9 except 3ACDT showing percent growth of 101.5. These compounds demonstrated cytotoxic effect on MCF-7 and SCC-40 cancer cell lines with the average cell growth percent values of 92.1 to 101.3 and 85.4 to 93.3 respectively at the same concentrations. Thus, the synthesized compounds are exceedingly active against HeLa and SCC-40 cell lines and moderately active against MCF-7.

The parameter GI50 was calculated using the graph of % control inhibition values and drug concentrations. The results are shown in Table 2.

| Cell Lines | MCF-7 | HeLa | SSC-40 |
|------------|-------|------|--------|
| Codes      | GI50* | GI50* | GI50*  |
| 3ACOT      | 53.6  | <10   | >80    |
| 3ACMT      | 61.5  | 13.8  | >80    |
| 3ACPT      | 63.1  | 40.8  | >80    |
| 3ACDT      | 25.4  | 70.6  | 44.1   |
| ADR        | <10   | <10   | <10    |
GI50 values are calculated for the 50% growth inhibition of cells. For getting the idea of the activity of the compounds the GI50 value of $\leq 10 \mu g/ml$ is considered as a good activity. It is observed that the GI50 value for 3ACMT is 13.8 $\mu g/ml$ against HeLa and other derivatives exhibit the GI50 values 40.8 and 70.6 $\mu g/ml$ for 3ACPT and 3ACDT respectively. The derivative 3ACOT shows the GI50 value comparable to standard ADR which is less than 10$\mu g/ml$. In terms of GI50 values, all the derivatives are active against human cervical cancer cell line (HeLa). 3ACDT is found to be most active against MCF-7 with GI50 value of 25.4 $\mu g/ml$.

2.3 Molecular Docking

Molecular docking study was performed to check the binding affinities of synthesized analogues with the Cyclooxygenase active site residues of COX-2 enzyme (PDB ID: 6COX) using the Auto Dock 4.2.3 software. A grid box covering the cyclooxygenase active site residues of the target protein was generated to induce the most effective conformational state of docking. Docking grid box size was set to 46×46×46 Å dimension and focused at 30.77, -1.64, 25 of X, Y and Z coordinates. Present docking study affirmed that all the four derivatives fit favorably into the cyclooxygenase active site of COX-2 displaying H-bonding interactions with CYS-41, CYS-47, ASN-34 and PRO-154 amino acid residues. The estimated free energy values of binding and the interacting amino acid residues are given in Table 3.

Table 3. Binding energy and the interacting amino acid residues

| Sr. No. | Compounds | B.E. kcal/mole | Binding amino acid Residue | Bond Lengths Å |
|---------|-----------|----------------|---------------------------|---------------|
| 1       | 3ACOT     | -9.6           | CYS-41, CYS-47            | 2.2, 3.5, 2.3 |
| 2       | 3ACMT     | -9.6           | CYS-41, CYS-47            | 2.1, 2.3      |
| 3       | 3ACPT     | -9.3           | ASN-34                    | 2.2, 2.4      |
| 4       | 3ACDT     | -10            | ASN-34, PRO-154           | 2.4, 3.3      |

The docking ribbon structures of 6COX protein with respective compounds are given in Fig. 5. The best binding energy was exhibited by 3ACDT followed by 3ACOT, 3ACTMT and 3ACPT respectively. 3ACDT shows hydrogen bonding interactions with two amino acid residues, viz. ASN-34 and PRO-154 in the protein cavity. The hydrogen bonding distances are 2.4 and 3.3 Å. The derivative 3ACOT shows affinity with two amino acid residues CYS-41 and CYS-47 with formation of 3 hydrogen bonds. These interactions cause stabilization of the compounds within the protein cavity. Based on these observations, compound 3ACDT has better stability within the 6COX protein cavity than other derivatives and contemplated to show enhanced anticancer activity.
3. Conclusions

Four 3-(2-(substituted-(trifluoromethyl)phenylamino)acetyl)-2H-chromen-2-one derivatives were prepared and tested for their anticancer activities against three cancer cell lines, human breast cancer cell line (MCF-7), human cervical cancer cell line (HeLa) and human oral squamous cell carcinoma (SCC-40). 3ACMT and 3ACDT derivatives exhibited good \textit{in vitro} anti-proliferative potency at lower concentrations against HeLa and MCF-7 cell lines while moderately active against SCC-40 only at higher concentrations. 3ACOT and 3ACMT express best activities against HeLa cell line at \( \leq 10 \mu g/ml \) concentration. The results are further supported by molecular docking study results which are in best agreement with experimental results. The observed anticancer activities can be accounted for the presence of polar interactions in between the synthesized compounds and amino acid residues in the protein. Such results further suggest that these new 3-(2-(substituted-(trifluoromethyl)phenylamino)acetyl)-2H-chromen-2-one derivatives may be promising compounds in the treatment of human cancers.

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4. Experimental

4.1 Materials and Methods: A. R. grade reagents were purchased from commercial sources Sigma-Aldrich and SD-FCL Chemical Limited, Mumbai, India and used without further purification unless otherwise stated. Solvents used for synthesis were dried by standard protocols. The MTT reagent was obtained from G-Biosciences, USA. All compounds were routinely checked for their purities by TLC on silica gel G plates using 20%-50% ethyl acetate in petroleum ether as solvent system and the developed plates were visualized by UV light. Melting points of synthesized compounds were recorded with open capillary tubes on a VEEGO melting point apparatus. The \(^1\)HNMR and \(^{13}\)CNMR were obtained from IISER, Bangalore, HRMS is obtained from NCL, Pune. IR spectra were recorded by “FT-IR JASCO” spectrometer from SPPU, Pune. The \textit{in vitro} anticancer activity was obtained from The Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai and ASR Lab, Abeda Inamdar Senior College, Pune.

4.2 Synthesis of 3-(2-phenylamino)acetyl-2H-chromen-2-ones derivatives: Four 3-(2-(substituted-(trifluoromethyl)phenylamino)acetyl)-2H-chromen-2-one derivatives (abbreviated as 3ACOT, 3ACMT, 3ACPT and 3ACDT) were synthesized using the methods explained by Guravaiah et al.\(^{40}\) The synthesis is shown in the following Scheme 2. A mixture of 3-(2-bromo acetyl)coumarin (a), Triflouro substituted anilines (b) and K2CO3 in 1:1:1 molar ratio was ground at room temperature for 8-10 minutes and monitored by TLC. After completion of the reaction, the mixture was treated with water and ethyl acetate. The solid separated was filtered and recrystallized using ethanol. Yield 92–96%.

![Scheme 2](https://example.com/scheme2.png)

\textit{Scheme 2.} Syntheses of 3-(2-(substituted-(trifluoromethyl)phenylamino)acetyl)-
4.3 SRB Assay: The *in vitro* anticancer testing was carried out using the SRB assay protocols. The test compounds were inoculated at 4 dose levels at 10, 20, 40, 80 μg/ml. Cell lines were counted, cultured and inoculated in 96 well plates. Each experiment was repeated three times. After incubation with different concentrations of test compounds, the cell cultures were stained with SRB dye. After washing with 1% acetic acid the protein bounded dye was extracted using Tris-HCl buffer base (100 μl, 0.01 M, pH 10.4). The optical density was determined on 96-well plate ELISA reader at 540 nm. Cell viability was expressed as a percentage growth of sample compounds with that of the control.

4.4 Cytotoxicity assay against non-cancerous cells: The cytotoxicity of synthesized analogues was performed on non-cancerous cells i.e. normal human peripheral blood mononuclear cells (PBMCs). The PBMCs were isolated from whole blood of human healthy volunteers using the procedure available in literature. PBMCs were seeded on 96-well microplates at a density of 1×10^5. Next day the culture medium was removed and cells were exposed to serial dilutions of the synthesized compounds in fresh culture medium. Cell proliferation was studied for 24-48 hours by means of MTT assay in which the yellow tetrazolium salt (MTT) is metabolized by viable cells to purple formazan crystals. The plates were incubated for 3 hours with MTT solution (5 mg/ml). Formazan crystals were dissolved in DMSO and the purple colour developed was observed spectrophotometrically at 570 nm wavelength using Readwell Touch Automatic Elisa Plate Reader (Robonik India Private Limited).

4.5 *In silico* Molecular Docking: Molecular docking studies were carried out on cyclooxygenase active site residues of COX-2 enzyme (PDB ID: 6COX) using the software, Auto Dock 4.2.6.14. The PDB file (6COX) of human cyclooxygenase enzyme was downloaded from Royal Society Protein Data Bank. The PDB was processed in Discovery Studio for removal of DNA and water molecules to make the binding sites free for interaction with the synthesized compounds. The cleaned up PDB files were used for generation of PDBQT file by adding Kollmann charges and further used for docking studies. The images were created with the help of Pymol Molecular Viewer software.

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