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Screening of 3-acetylcoumarin derivatives as multifunctional biological agents

Anees Pangal[a,c], Pranav Tambe[b,c] and Khursheed Ahmed[a,c*]

[a]*Department of Chemistry & Post Graduate Centre, Abeda Inamdar Sr. College of Arts, Science & Commerce (Autonomous), Camp, Pune – 411001, India*
[b]*Department of Biotechnology, Abeda Inamdar Sr. College of Arts, Science & Commerce (Autonomous), Camp, Pune – 411001, India*
[c]*Advanced Scientific Research Laboratory, Abeda Inamdar Sr. College of Arts, Science & Commerce (Autonomous), Camp, Pune – 411001, India*

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A B S T R A C T

In this work, the multifunctional potential of four 3-acetylcoumarin derivatives was studied. The derivatives were significantly active against bacteria *Staphylococcus aureus*, *Pseudomonas aeoginosa* and fungal strain *Candida albicans*. The results of antioxidant activity assays were promising when compared to ascorbic acid. The *in vitro* anticancer activity was carried out using MTT assay on human cancer cell line COLO-205 and 3ACDT showed commendable antiproliferative activity along with appreciable tumor selectivity with distinct selectivity index. Moreover, ADMET properties of the compounds were determined using the pKCSM and SwissADME online tools and all compounds were found with good pharmacokinetic profile. Hence, from the obtained results from all the 3-acetylcoumarin derivatives, 3ACDT exhibited good therapeutical potential and can be optimized for lead development.

1. Introduction

In recent years, the strategy of making hybrids of two or more than two biologically active motifs has emerged as a popular approach that involves conglomeration of two or more pharmacophores in one molecular scaffold to develop multifunctional molecules.[1] The hybrid structure is expected to exhibit multiple biological activities, modified selectivity profile, different or dual modes of action with reduced undesired side effects due to mixing of pharmacophores in one molecule. Such molecules may be further modified to explore favorable pharmacokinetics and oral bioavailability.[2,3] Using this approach, several research groups have designed and synthesized many hybrid molecules. Some prominent examples of such molecules include ziprasidone, duloxetine and ladostigil for multifactorial CNS diseases, and sunitinib and lapatinib for treatment of cancers.[4] A lot of hybrid molecules for treatment of other multifactorial diseases that are highly variable and heterogeneous involving multiple organ systems and targets such as metabolic disorders, malaria, inflammation, organophosphorous poisoning and ischaemia have been reported.[5]

Emergence of drug resistance in pathogenic microorganisms is a serious challenge, which has aggravated further in recent times. Regrettably, with any new generation of antibiotics or newly introduced drugs we witness the appearance of the newer resistant strains of microorganisms.[6] Currently, drug-resistant infections pose a serious health risk to individuals. Many lives are lost every year owing to infections which can no longer be treated with existing drugs. Discovery and development of new antibiotics is essential to kill drug-resistant microorganisms.[7] Increasing incidences of microbial

* Corresponding author.
E-mail address khursheedahmed@azamcampus.org (K. Ahmed)

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infection is a major health concerns which is usually caused by genomic mutations or development of an evolved mechanism against drug action.[8] The synergistic action of various pharmacophores incorporated in a hybrid structure can help to increase the efficacy of molecule against resistant strains because of its ability to attack multiple targets.[9] Therefore, hybrid molecules can reduce the risk of multidrug resistance and drug interactions, which may help overcome microbial resistance.[10]

The main feature of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species enter biological systems from a wide variety of sources. These free radicals may initiate degenerative disease through the oxidation of nucleic acids, proteins, lipids and DNA. Phenolic acids, polyphenols and flavonoids are natural antioxidant compounds that inhibit the oxidative mechanisms that lead to degenerative diseases by scavenging free radicals such as peroxide, hydroperoxide or lipid peroxyl.[11] A wide range of antioxidants both synthetic and natural have been utilized in the treatment of human diseases.[12]

Anti-cancer drugs have traditionally been designed to damage the abnormally dividing cell by interrupting the cell division process.[13] The cell cycle can be affected by cyctotoxic agents like DNA intercalating agents (*e.g.* adriamycin), DNA cross-linking agents (*e.g.* cis-platin), topoisomerase inhibitors (*e.g.* campothecins), cytoskeleton-disrupting agents (*e.g.* vinblastin) and antimetabolites (*e.g.* mercaptopurine). Though these drugs are effective, they are cytotoxic towards normal proliferating tissues such as the haematopoietic system. These agents are therefore carefully administered to allow recovery of normal but not malignant cells from drug exposure.[14]

Coumarin compounds and the other organic molecules are very important due to their various uses as reported before.[15-20] Coumarins or 2*H*-1-benzopyran-2-one are a class of phenolic substances found as secondary metabolites in plants, microbes, and are widely used as additives in cosmetics, food, perfumes and drugs.[21] Coumarins have diverse pharmacological activities (**Fig. 1**) like anti-inflammatory (e.g. Esculetin),[22] antibacterial (e.g. Ammoresinol, Novobiocin, Coumermycin),[6] anti-TB and antimycobacterial (e.g. Ostruthin),[23] antihypertensive (e.g. Imperatorin),[24] antihyperglycemic (e.g. Umbelliferone),[25] blood anticoagulant activity (e.g. Warfarin, Dicoumarol)[26] and anticancer activity.[27-29]

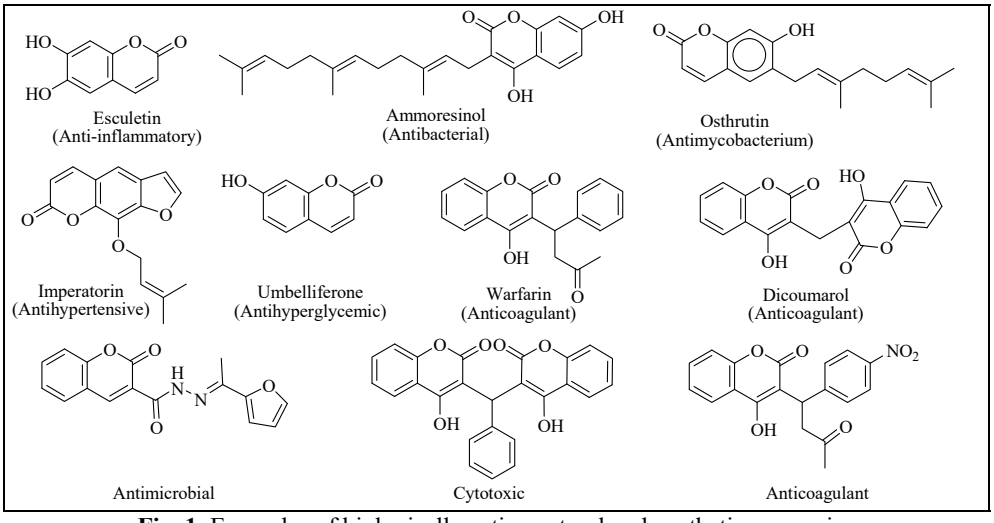


Fig. 1. Examples of biologically active natural and synthetic coumarins

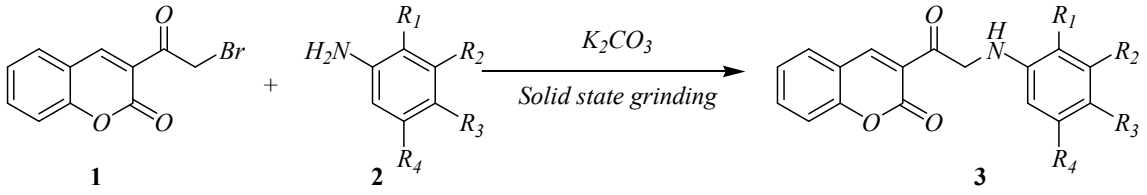
We have previously reported the synthesis and anticancer studies of 3-(2-(subsituted-(trifluoromethyl)phenylamino)acetyl)-2H-chromen-2-one derivatives.[30] The present work mainly focuses on determining the multifunctional potential like antimicrobial, antioxidant and antiproliferative studies of these derivatives. The antimicrobial activity was tested on *Staphylococcus aureus* NCIM 5021, *Pseudomonas aeruginosa* NCIM 5029, and *Candida albicans* NCIM 3100. The antioxidant was evaluated using DPPH Scavenging Activity and the antiproliferative activity was tested on human colon carcinoma cell lines (COLO-205) by MTT assay. Further, *in-silico* studies of these analogs were determined to understand ADMET and physicochemical properties, and to examine the drug likeness of the analogs using online sources. The derivative 3ACDT was found to be more effective biological agent among the synthesized analogs.

2. Results and Discussion

2.1 Chemistry

We have effectively synthesized new 3-acetylcoumarin and trifluoromethylaniline analogs which are abbreviated as 3ACOT, 3ACMT, 3ACPT and 3ACDT in greater yields. 3-acetylcoumarin was brominated with Br_2 in chloroform to obtain 3-(bromoacetyl)coumarin **(1)**. The compound 3-(bromoacetyl)coumarin **(1)** was ground with substituted trifluoromethyl

anilines **(2)** and K_2CO_3 in a mortar and pestle to obtain acetylcoumarin derivatives **(3)**. The reaction was monitored by TLC using petroleum ether/ethyl acetate (7:3 by V/V) as a solvent system and the developed plates were visualized in UV light and iodine vapours. The crude products were recrystallized from ethanol **(Fig. 2)**.



where-		R_1	R_2	R_3	R_4	Yield (%)	M. P. (°C)
	3ACOT	CF_3	H	H	H	90	205-206
	3ACMT	H	CF_3	H	H	92	198-200
	3ACPT	H	H	CF_3	H	92	210-212
	3ACDT	H	CF_3	H	CF_3	90	204-206

Fig. 2. Synthesis of 3-acetylcoumarin derivatives **(3).**

The structures of 3-acetylcoumarin derivatives **(3)** were confirmed by spectral data like HRMS, FTIR, ^{1}H-NMR and ^{13}C-NMR. The HRMS (EI) spectra of the synthesized derivatives showed major peaks corresponding to the expected M+1 fragment at 348.08, 348.08, 348.08 and 438.05 respectively. In the IR spectra of these analogs one peak each between 3020 to 3047 cm^{-1} for the –NH, 1714 to 1724 cm^{-1} for the >C=O of lactone moiety and 1675 to 1686 cm^{-1} for the >C=O of ketonic moiety respectively were observed. The ^{1}H-NMR spectrum of the synthesized analogs showed –CH_2 protons at 4.7 ppm and a sharp singlet at 8.6 ppm is observed for the olefinic proton present on C-4 of the coumarin ring. The aromatic hydrogen atoms were present in the range of 6.7 to 7.7 ppm. On the other hand, the protons of –NH group appeared as a singlet at 2.17 ppm. In the ^{13}C-NMR of these analogs, the signals for aromatic carbon atoms appeared at 109.3 to 149.6 ppm, the ketonic and lactone carbons at about 185.8 to 192.7 and 155.4 to 158.9 ppm respectively. In all these compounds the methylenic carbon showed a signal in the range of 35 to 54 ppm.

2.2 Antimicrobial activity

The 3-acetylcoumarin derivatives **(3)** were tested for antibacterial and antifungal activity using *Staphylococcus aureus* NCIM 5021, *Pseudomonas aeruginosa* NCIM 5029 and *Candida albicans* NCIM 3100 strains. The results were compared with the standard drugs viz. streptomycin (antibacterial) and itraconazole (antifungal). Growth curves are shown in **Fig. 3**. Each compound showed reasonable antimicrobial activity in a dose-dependent way. 3ACDT has the most active bacterial and fungal strains. At higher concentrations, the analogs showed maximum potential and killed more than 90% of the bacteria and fungus.

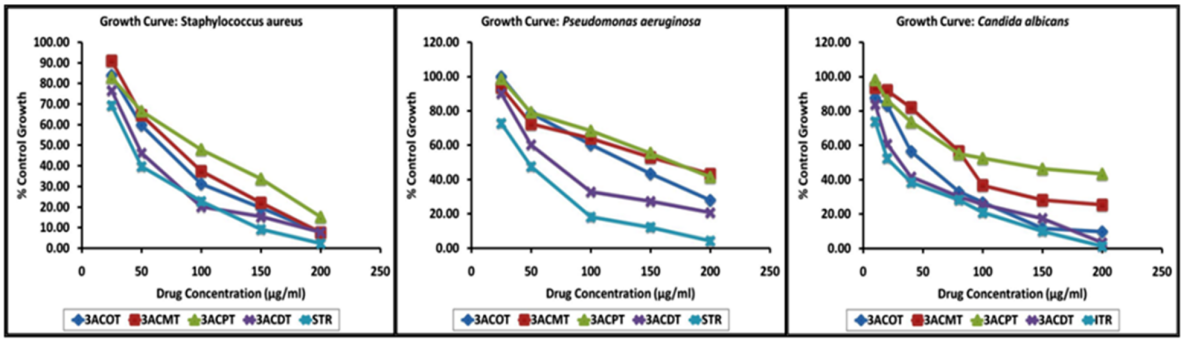


Fig. 3. Results of Antimicrobial activity

IC_{50} was calculated and tabulated in **Table 1**. In terms of the IC_{50} value, all the analogs were good to moderate anti-microbial agents. The potent analog, 3ACDT exhibited the IC_{50} value of 58.60 ± 4.23 µg/ml while other analogs showed the IC_{50} value between 82 to 104 µg/ml which is a slightly higher concentration than the value of streptomycin against *Staphylococcus aureus.* The same analog, 3ACDT was found to be the most potent candidate among others and exhibited the IC_{50} value of 95.21 µg/ml and exhibited moderate anti-bacterial potential compared with the standard drug against *Pseudomonas aeruginosa*. The derivative 3ACDT showed significant activity against *Candida albicans* comparable with the itraconazole standard and other derivatives expressed weak antifungal potential. The analog 3ACDT was found to be a good antimicrobial agent against all three microorganisms with good IC_{50} values in comparison with standards. However,

the analog, 3ACDT possessed good antimicrobial activity but from the IC_{50} values, it is evident that this analog is more potent against *Candida albicans* and can be selected for further development and optimization as an antimicrobial agent.

Table 1. IC_{50} concentration values of 3-acetylcoumarin derivatives

Microorganism Compound	*Staphylococcus aureus*	*Pseudomonas aeruginosa* IC_{50} Drug Concentrations (µg/ml)	*Candida albicans*
3ACOT	82.42 ± 4.83	136.15 ± 10.98	72.01 ± 0.47
3ACMT	93.34 ± 1.73	164.25 ± 0.90	108.98 ± 0.51
3ACPT	104.09 ± 3.16	167.89 ± 5.60	141.06 ± 1.97
3ACDT	58.60 ± 4.23	95.21 ± 3.42	51.32 ± 3.89
STR	44.75 ± 446	54.17 ± 7.64	--
ITR	--	--	32.82 ± 0.82

2.3 Antioxidant activity

The 3-acetylcoumarin derivatives **(3)** were evaluated for antioxidant activity using the DPPH radical scavenging method. 50µl samples (1 to 150 µg/ml) and 150 µl DPPH solution were mixed in 96 well plates and incubated for 30 minutes in the dark. After 30 minutes the absorption was measured at 520 nm. The scavenging activity versus concentration of the compounds was plotted and presented in **Fig. 4**. The IC_{50} concentrations were determined from an online source (http://www.ic50.tk) and the results obtained were compared with the IC_{50} value of ascorbic acid (Standard/STD). The results revealed that 3ACMT and 3ACDT among the compounds were highly active. The other derivatives showed good antioxidant activity when compared with the standard. The scavenging effect increased with the increasing concentrations of test compounds. The calculated IC_{50} values for standard drug and the synthesized derivatives, 3ACOT, 3ACMT, 3ACPT and 3ACDT were 8.25 ± 1.72, 25.81 ± 0.90, 12.32 ± 0.30, 17.74 ± 0.25 and 12.18 ± 0.93 µg/ml, respectively. Among these derivatives, 3ACDT exhibited the best antioxidant activity.

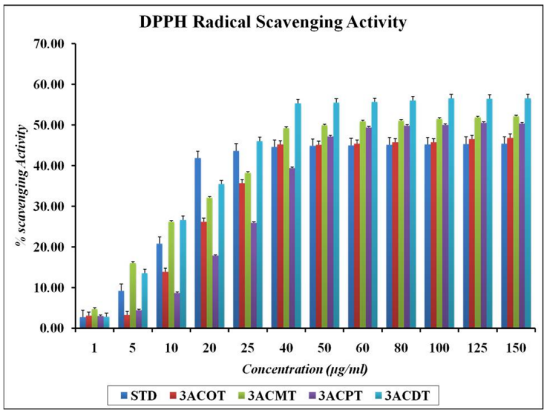


Fig. 4. Results of Antioxidant activity

2.4 Anticancer activity

The anti-proliferative activity of the 3-acetylcoumarin derivatives was tested against human colon carcinoma cell line (COLO-205) by MTT method. All experiments were performed in triplicates. The results are expressed as the percent growth (%) at different concentrations of test compounds. 5-fluorouracil (5-FU) was used as standard. The concentration of drug causing 50% inhibition of cell growth was characterized by IC_{50} value and is depicted in **Table 2**. The graph of the dose-dependent effects of the derivatives on cancer cell lines is shown in **Fig. 5**.

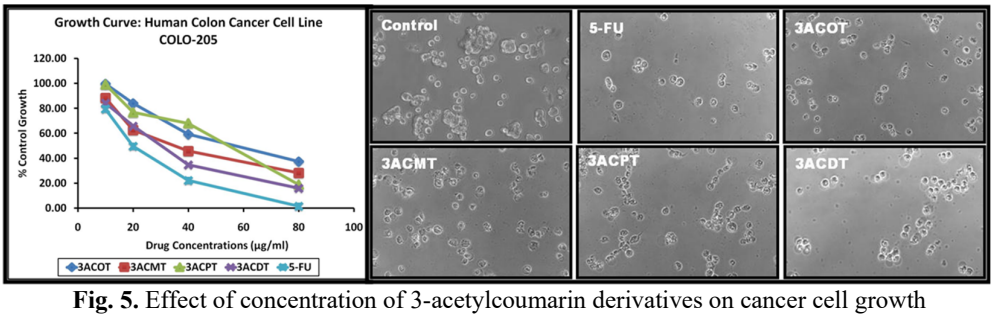


Fig. 5. Effect of concentration of 3-acetylcoumarin derivatives on cancer cell growth

It is apparent from the results that with an increase in the concentrations of the tested compounds, the anti-proliferative potential of the hybrids increases, too. All derivatives exhibited the dose-dependent antiproliferative potential against COLO-205 cells. The IC_{50} value of 3ACDT was comparable with the standard on COLO-205 cells. The IC_{50} value of 37.76 ± 0.37 µg/ml was observed for 3ACDT, making these compounds relevant for further lead development.

Table 2. IC_{50} values (µg/ml) and Selectivity Index (SI) of 3-acetylcoumarin derivatives

	IC_{50} Drug Concentrations		Selectivity Index (SI)
Cell Lines	COLO-205	PBMC	COLO-205
3ACOT	60.93 ± 1.07	287.18 ± 3.85	4.71
3ACMT	45.73 ± 1.60	203.17 ± 6.04	4.44
3ACPT	52.23 ± 0.30	204.69 ± 3.06	3.92
3ACDT	37.76 ± 0.37	223.86 ± 3.10	5.93
5-FU	26.02 ± 0.75	---	---

The activities of test compounds were compared with those of non-malignant normal human peripheral blood mononuclear cells (PBMCs). More than 75% of the cell growth was found on treatment with highest concentration of the compounds and this concentration is far higher than IC_{50} value of the compound against cancer cell lines. This selective activity against cancer cells by these derivatives though at a higher concentration, is commendable for anticancer activity. Additionally, Results from both cancerous and normal cells were then compared to determine the selective activity. The selectivity index for cancer cell lines was calculated from the ratio of their respective IC_{50} values to the IC_{50} values of PBMCs **(Table 2)**. The selectivity index indicates the selectivity of a given compound between normal and cancer cells. The higher the magnitude of the selectivity index, the greater is its selectivity.[33] The highest SI was exhibited by the analog 3ACDT (5.93) compared to the other analogs. In general, all the synthesized derivatives showed a good SI (more than 2) and the selectivity indexes entail that the synthesized compounds were more selective towards cancer cells than the non-malignant cells.

2.5 ADMET Properties and Drug Likeliness

ADMET Predictor is a computer program designed for estimating pharmacokinetic parameters or properties of drug-like compounds from their molecular structures. Being highly bioactive and less toxic is not good enough for a drug-like compound to qualify as a good drug candidate. A better profile of pharmacokinetic is exclusively important for a novel compound that should be examined in the process of drug or druglike compounds discovery. Hence, it is very significant to pre-evaluate the ADMET profile of new compounds to avoid waste of time and resources.[34] Hence, to make sure that the synthesized compounds show the potential of a drug, we predicted the pharmacokinetic profile and druglike properties of the new hybrid compounds using the online sources pkCSM and SwissADME and the results are shown in **Table 3**.

Table 3. ADMET properties of synthesized 3-acetylcoumarin derivatives

Property	Model Name	3ACOT	3ACMT	3ACPT	3ACDT
Absorption	Water solubility[a]	-4.453	-4.405	-4.405	-5.37
	$CaCO_2$ permeability[b]	1.444	1.44	1.441	1.063
	Intestinal absorption (human)[c]	91.21	90.62	90.66	88.14
	Skin Permeability[d]	-2.817	-2.813	-2.817	-2.793
Distribution	VDss (human)[e]	-0.158	-0.219	-0.21	-0.246
	BBB permeability[f]	0.165	0.131	0.159	0.06
	CNS permeability[g]	-1.785	-1.788	-1.786	-1.64
Metabolism	CYP2D6 inhibitor[h]	No	No	No	No
	CYP3A4 inhibitor[h]	Yes	Yes	Yes	Yes
Excretion	Total Clearance[i]	0.198	0.191	0.182	-0.156
	Oral Rat Acute Toxicity[j]	2.681	2.691	2.708	2.753
	Oral Rat Chronic Toxicity[k]	0.935	0.912	0.872	0.673
	Hepatotoxicity[h]	No	No	No	No
	Skin Sensitization[h]	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](LD50 in mol/kg), [k](LOAEL in log mg/kg_bw/day)

The determined values showed that the molecules have a great solubility potential in the water and the solubility values between -4.405 to -5.37 mol/L. The values 1.063 to 1.444 indicate good Caco-2 permeability. Drugs with less than 30% intestinal absorption are considered to be poorly absorbed in the intestine. The compounds exhibited a good percent of intestinal absorption ranging from 88.14 to 91.21% while the compounds showed low skin permeability. The distribution volume (VDss), blood-brain barrier membrane permeability (logBB) and CNS permeability were used to characterize the distribution of compounds. The distribution volume of 3ACMT, 3ACPT and 3ACDT is relatively low in comparison with other analog 3ACOT while BBB permeability in the range of 0.06 to 0.165 which <0.3 and >-1 suggest difficulty crossing the blood–brain barrier. For CNS permeability, the logPS is < -3 which suggests the ability of the compounds to penetrate the CNS. This ability of the analogs to disallow the metabolism of xenobiotics in the body is due to inhibition of CYP3A4 enzyme. The values of total clearance exhibited by these analogs imply that the molecule would not accumulate in the body. All compounds have LD50 values greater than 0.5mM and are non-toxic. Exposure to low to moderate doses of drugs over

long periods is of significant concern in many treatment strategies. Chronic studies aim to identify the lowest dose of a compound that results in an observed adverse effect (LOAEL). All the derivatives show LOAEL in the range of 0.673 to 0.935. The predicted results also show that these analogs are non-hepatotoxic and don't have skin sensitization. Hence, the results of ADMET studies revealed that the compounds have good ADMET and pharmacokinetic properties.

The physicochemical properties give a global description of the structures[35] of analogs such as molecular weight, molecular refractivity, topological polar surface area, number of rotatable bonds, heavy atoms, and hydrogen bond acceptors and donors (**Table 4**). The bioavailability properties shown by the analogs are within the range to be a good drug candidate. Therefore, we can conclude that these compounds possess good pharmacological properties.

Table 4. Physicochemical properties of 3-acetylcoumarin derivatives

Properties		3ACOT	3ACMT	3ACPT	3ACDT
Molecular weight		347.29	347.29	347.29	415.29
No. of Heavy atoms		25	25	25	29
No. of Arom. heavy atoms		16	16	16	16
No. of Rotatable bonds		5	5	5	6
No. of H-Bond acceptors		6	6	6	9
No. of H-Bond donors		1	1	1	1
Molar Refractivity		86.51	86.51	86.51	91.54
Total polar surface area Å²		59.31	59.31	59.31	59.31
Solubility	Log S (ESOL)	-4.91	-4.91	-4.91	-5.75
	Log S (Ali)	-5.36	-5.36	-5.36	-6.28
	Log S (SILICOS-IT)	-7.38	-7.38	-7.38	-8.21
Lipophilicity	MLOGP	2.90	2.90	2.90	3.72
	WLOGP	5.07	5.07	5.07	7.24
	XLOGP3	4.40	4.40	4.40	5.28

The drug likeness was established based on the physicochemical properties to find oral drug candidates. Five different rule-based filters are commonly used to determine the drug likeness of any compound.[36] The result of drug likeness evaluation of analogs is shown in **Table 5**.

Table 5. Drug Likeness evaluation of 3-acetylcoumarin derivatives

Rule-based filters	3ACOT	3ACMT	3ACPT	3ACDT
Lipinski violations	0	0	0	0
Ghose violations	0	0	0	1 (WLOGP>5.6)
Veber violations	0	0	0	0
Egan violations	0	0	0	1 (WLOGP>5.88)
Muegge violations	0	0	0	1 (XLOGP3>5)
Bioavailability Score	0.55	0.55	0.55	0.55
PAINS No. of Alerts	0	0	0	0
Brenk No. of Alerts	1 alert (Coumarin)	1 alert (Coumarin)	1 alert (Coumarin)	1 alert (Coumarin)
Lead likeness No. of Violations	1 (XLOGP3>3.5)	1 (XLOGP3>3.5)	1 (XLOGP3>3.5)	2 (MW>350, XLOGP3>3.5)

The predictions revealed that all test compounds have good drug similarity and can be suitable drug candidates for further study. A tested molecule can only be orally active or absorbed if it does not violate any two or more of the above rules. All the synthesized analogs are in agreement with Lipinski's rule and Veber's rule. According to Ghose's rule, all the compounds pass this rule excluding 3ACDT due to the lipophilicity parameter i.e. WlogP which is more than 5.6. According to Egan's rule and Muegg's rule, all the compounds are in agreement with the two rules excluding one compound, 3ACDT, due to lipophilicity parameters like WlogP and XlogP3 are more 5.88 and 5.6 respectively. All molecules resist Brenk's rule due to the coumarin fragments. These preliminary results provide the lead for the design of more potent biological drugs with lesser toxicity.

3. Conclusion

Four 3-acetylcoumarin derivatives were tested for in vitro antimicrobial, antioxidant and anticancer activities. The results revealed that the derivative 3ACDT was more potent antimicrobial agents as compared to other analogs. 3ACDT exhibited the best antioxidant activity and it may have a better therapeutic potential as a chemoprotective agent. The cytotoxicity of the compounds was tested on human peripheral mononuclear cells (PBMCs) and they were found non-toxic to the normal cells. 3ACDT exhibited good anti-proliferative potential against cancer cell line COLO-205 with remarkable IC_{50} value making this derivative a choice of interest for further lead development. Further, all the derivatives showed a good selectivity index (more than 2) and the selectivity indexes entail that the 3-acetyl derivatives were more selective towards cancer cells than the non-malignant cells. It is appropriate to mention one important observation about antibiotics, which is their capacity to interfere with the normal functional of living cells.[37] This property on one hand is useful for controlling the growth of microorganism but at the same time, they can act as double-edged sword and can also interfere

with the functioning of metabolically active, fast multiplying cancer cells.[38] The compounds in the present work have shown activity against the cancer cell lines as well as against the microorganisms. The observed antioxidant property of these compounds provides them the advantage of reducing the oxidative stress and plays the role of chemoprotective agents in the biological system by protectecting the cells from oxidative stress-induced neoplastic changes. The predicted ADMET, pharmacokinetic properties and drug likeness via *in silico* methods showd that these compounds are pharmacokinetically compatible. The compounds showed a high level GI absorption up to 91.21% and the molecules have a great solubility potential in water. The VDss, BBB membrane permeability (logBB) and CNS permeability were used to characterize the distribution of compounds and all these analogs showed a good distribution in the body. All the synthesized analogs were unable to inhibit CYP2D6 but they were found to be good CYP3A4 inhibitors depicting the ability of these analogs to metabolize the xenobiotics in the body. The predictions also showed non-toxic nature of these analogs, reflected through high values of the total clearance, non-hepatotoxic nature and no skin sensitization. These results of ADMET studies revealed that the compounds have acceptable ADMET and pharmacokinetic properties. These preliminary results provide the lead for the design of more potent and selective biological agents with less toxicity.

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4. Experimental

4.1 Materials and Methods

A. R. grade reagents were purchased from commercial sources and were dried by standard protocols. The starting materials such as 3-(2-bromoacetyl)coumarin and substituted anilines were procured from Sigma-Aldrich, Mumbai, India. Solvents like acetone, methanol, ethanol and dichloromethane were obtained from SD-FCL Chemical Limited, Mumbai, India. 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 were determined with open capillary tube on a VEEGO melting point apparatus. The HRMS, FTIR and NMR spectroscopic data were obtained from CIF, Savitribai Phule Pune University, Pune and CIF, IISc, Bangalore. The *in vitro* biological activities and *in silico* predictions were carried out at Advanced Scientific Research Laboratory, Abeda Inamdar Senior College, Pune.

4.2 General procedure for the synthesis

The procedures used for synthesis of acetylcoumarin derivatives and detailed physical properties and spectral characterization data is mentioned in our previous paper.[30]

4.3 Antimicrobial Activity

Nutrient broth was prepared and autoclaved for 20 minutes at 120 psi. Cultures of *Staphylococcus aureus* NCIM 5021 and *Pseudomonas aeruginosa* NCIM 5029 strains were inoculated and incubated at 37^{O}C for 24. Then, each of 96 well plate was inoculated with a microbial inoculums. After dilution of standardized microbial suspension adjusted to 0.5 McFarland scale (10^8 CFU/mL). 180 µl of cell suspension was seeded in each well and 20µl of different concentrations compounds and standard. Streptomycin was used as standard. After shaking the plates were incubated at 37 ^{O}C for 24 hr. After incubation, the absorbance of each well was recorded at 620 nm using Readwell Touch Automatic Elisa Plate Reader (Robonik India Private Limited). All the experiments were performed in triplicates and the percentage growth was calculated using the formula –

$$Percent\ Grwoth\ Inhibition = \frac{Ti}{C} \times 100$$

where, Ti = Growth of the microorganisms in the presence of drug and C = Control growth.

Similar procedure was followed for antifungal activity with slight changes. The Potato dextrose broth (PDB) was used for fungal culture pre enrichment, dilutions and inoculations. The antifungal activity was evaluated on *Candida albicans* NCIM 3100. 180 µl of the cell suspension was seeded in each of 96-well plates and 20 µl of different concentrations of compounds and standard was added to each well in the plate. Itraconazole was used as standard. After well-mixing, the 96-well plates were incubated at 37 ^{O}C for 24 hr. After incubation, the absorbance of each well was recorded at 620 nm using Readwell Touch Automatic Elisa Plate Reader (Robonik India Private Limited). All the experiments were performed in triplicates and the percentage growth was calculated using the above formula.

4.4 Antioxidant Activity (DPPH Scavenging Activity)

DPPH free radical scavenging activity was determined using the microplate assay standard method with slight modifications. Drug stock solution (1000 µg/ml) was diluted to final concentrations of 1, 5, 10, 20, 25, 40, 50, 60, 80, 100, 125 and 150 µg/ml in methanol. 0.004 g DPPH reagent was dissolved in 100 ml methanol. 50 µl sample and 150 µl DPPH solution were added. The plates were incubated for 30 min at room temperature in the dark, and absorbance (Abs) was recorded at 520 nm wavelength with a microplate reader (Readwell Touch Automatic Elisa Plate Reader (Robonik India Private Limited) and converted into the percentage antioxidant activity. Ascorbic acid was used as standard. The percent radical scavenging was calculated from the absorbance using the following formula –

$$\%\ \text{Radical scavenging activity} = \frac{(\text{Abs of control} - \text{Abs of sample})}{(\text{Abs of control})} \times 100$$

4.5 Cell Viability Inhibition Assay (MTT Assay)

The *in vitro* antiproliferative activity of compounds was evaluated against human colon carcinoma cell lines (COLO-205) using MTT assay protocol. 5-Fluorouracil (5-FU) was used in this test as a positive control. The tested cell lines were purchased from The National Centre for Cell Science (NCCS), Pune and harvested on the appropriate growth medium. The growth medium was supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of heat-inactivated fetal bovine serum in a humidified 5% (v/v) CO_2 atmosphere at 37 ^{O}C. Then 1x10^6 cells per well from the cancer cell lines were seeded into each well and replaced 24-48 hours later with fresh medium containing different dilutions of the compounds diluted using the DMEM. After 48 hours, 5% MTT solution was added and incubated further for 4 hours. MTT formazan formed by metabolically viable cells was dissolved in DMSO, and after 10-20 minutes, absorbance was recorded at 570 nm using Readwell Touch Automatic Elisa Plate Reader (Robonik India Private Limited). Metabolic viability of different cancer cell lines treated with test compounds were compared with corresponding cells without any treatment (taken as 100% viable). All the experiments were performed in triplicates and the percentage growth was calculated using the formula –

$$Percent\ Grwoth\ Inhibition = \frac{Ti}{C} \times 100$$

where, Ti = Growth of the microorganisms in the presence of drug and C = Control growth.

4.6 In vitro cytotoxicity assay against non-cancerous cells

To study the toxicity against normal cell lines, the synthesized analogues were tested against non-cancerous normal human peripheral blood mononuclear cells (PBMCs). Isolation of peripheral blood mononuclear cells was done using Ficoll-Hypaque according to the method[31] and MTT cytotoxic assay was used to evaluate the cytotoxicity of the compounds. 1×10^6 cells per well were seeded in a 96-well plate, then exposed to different concentrations of compounds for 48 hours along with a control well. After 48 hours, the culture medium was removed by washing with PBS. 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). Furthermore, the selectivity index was calculated by using the following formula.

$$\text{Selectivity Index} = \frac{\text{IC50 of a compound in normal cells}}{\text{IC50 of a same compound in cancer cells}}$$

4.7 ADMET and pharmacokinetic studies

ADMET and pharmacokinetic properties were checked according to the method[32] 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. The physicochemical properties and drug likeness of the synthesized analogs was evaluated using an online source SwissADME (link: http://www.swissadme.ch/).

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