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ORIGINAL ARTICLE

Antioxidant Activities and Cytotoxicity of Selected Coumarin Derivatives: Preliminary Results of a Structure–Activity Relationship Study Using Computational Tools

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Purpose: In the present investigation a series of coumarin derivatives (CDs) were evaluated for their *in vitro* antioxidant and anticancer activities. This study was to assess the suitability of a series of structurally different CDs as possible antioxidant and anticancer agents.
Methods: The antioxidant studies were carried out using a 2,2,-diphenyl-1-picrylhydrazine (DPPH) radical scavenging assay, while the anticancer activity was assessed by performing the (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliuum bromide-based (MTT-based) cytotoxicity assay using different cancer cell lines. The physico-chemical properties of the test CDs related to free radical scavenging reactions and other biological properties were also calculated using BioMed Cache 6.1.10.
Result: Selected CDs showed significant cytotoxicity against different cancer cell lines in an IC_{50} range of 7.51–17.48 μM. All the selected CDs were demonstrated to have considerable concentration-dependent DPPH radical scavenging activity.
Conclusion: These results may signify the importance of selected CDs as antioxidant and anticancer agents.

1. Introduction

Coumarin (2H-chromen-2-one) and its derivatives are widely distributed in nature and exhibit a broad pharmacological profile. Coumarin derivatives (CDs) are often discussed because of their diverse biological properties. A vast body of literature has accumulated recently linking the role of coumarin with several bioactivities, including: anticancer,[1] anticoagulant, estrogenic, dermal, photosensitizing, antimicrobial, vasodilator, molluscacidal, antihelminthic, sedative, hypnotic, analgesic, hypothermic[2,3] and free radical scavenging activity especially the superoxide anions generated by activated neutrophils.[4] CDs have attracted considerable attention from organic and medicinal chemists, as they are widely used as fragrances, pharmaceuticals and agrochemicals.[2] Their antioxidant, bacteriostatic and anti-cancer activities make these compounds attractive for investigators for further backbone derivatization and screening as novel therapeutic agents and other foremost topics of this field of research.[5,6] Some reports have emphasized the efficacies of pure coumarins against Gram-positive and Gram-negative bacteria as well as fungi.[7] In addition CDs have been shown to inhibit cell proliferation in a cancerous cell line.[8]

Experimental investigations as well as clinical and epidemiological findings have provided evidence supporting the role of reactive oxygen metabolites or free radicals in the etiology of cancer.[9] Certain aldehydes such as malonyldialdehyde, the end product of lipid peroxidation arising from free radical degeneration of polyunsaturated fatty acids, can cause cross-linking in lipids, proteins and nucleic acids leading to cellular damage. The human body is equipped with certain enzymatic and nonenzymatic antioxidants which can counteract the deleterious actions of free radicals and radical-induced cellular and molecular damage.[10] Disruption of this sensitive balance between the free radicals and the antioxidants may lead to cellular damage and trigger carcinogenesis.[11] The circumstantial literature concerning CDs cited above inspired us to undertake the present studies on selected CDs and evaluate them as possible antioxidant and anticancer agents. Different physicochemical descriptors have been calculated *in silico* to discuss the possible structure–activity relationship.

2. Materials and methods

2.1. Chemicals

The selected CDs (Figure 1, C1–C15), 2,2,-diphenyl-1-picrylhydrazine (DPPH), 3- and (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliuum

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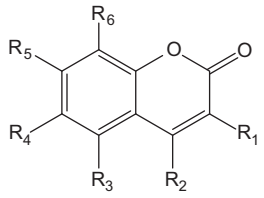

Structure number	R_1	R_2	R_3	R_4	R_5	R_6
C1	OH	H	H	H	H	H
C2	H	OH	H	H	H	H
C3	H	H	H	OH	H	H
C4	H	H	H	H	OH	H
C5	H	H	H	H	OH	OH
C6	H	CH_3	H	H	OH	OH
C7	H	H	H	H	OCH_3	H
C8	H	OH	H	CH_3	H	H
C9	H	CH_3	H	OH	H	H
C10	H	H	CH_3	H	OH	H
C11	H	CH_3	OH	H	OH	H
C12	H	OH	H	CH_3	CH_3	H
C13	CH_3	CH_3	H	H	OH	CH_3
C14	C_6H_5	CH_3	H	H	OH	H
C15	C_6H_5	OH	OH	H	OH	H

Figure 1 Chemical structures of selected coumarin derivatives.

bromide (MTT) were purchased from Sigma-Aldrich Co, (St Louis, MO, USA), cancerous cell lines such as HeLa-B75 (cervix cancer), HEP-3B (liver cancer), and HL- 60 (leukemia) were obtained from the National Centre for Cell Science, Pune (Maharashtra, India). All other reagents and solvents used were obtained from commercial sources and were of analytical grade.

2.2. Antioxidant activity of coumarin derivatives

DPPH free radical scavenging activity of selected CDs was assessed using the previously reported method.[12] In brief, the reaction cocktail contained equal volumes of DPPH solution (10^{-4} M, in absolute ethanol) with individual concentrations of selected CDs (10–30 μM). After 20 minutes of incubation the mixtures were read at 517 nm using an Automatic Ex-Microplate Reader (M 51118170) (Thermo, Vantaa, Finland). Ascorbic acid was used as a reference compound.

2.3. Cytotoxicity assay

The MTT-based cytotoxicity assay was performed using an earlier reported method.[13] Cells were harvested and inoculated ($4.5–5.0 \times 10^4$ cells/well) in 96-well microtiter plates. Cells were washed with phosphate-buffered saline (PBS) and then inoculated with and without the CDs. After 72 hours of incubation, the medium was aspirated followed by addition of 10 μL of MTT solution (5 mg/mL in PBS, pH 7.2) to each well and the plates were incubated for 4 hours at 37 °C. After incubation, 100 μL dimethyl sulfoxide was added to the wells followed by gentle shaking to solubilize the formazan dye. The absorbance of the mixture was read at 540 nm and the surviving cell fraction was calculated. Methotrexate was used as a reference compound.

2.4. Calculation of quantum chemical descriptors

The log P values, molecular weight and quantum chemical descriptors like E_{HOMO} (Energy of Highest Occupied Molecular Orbital), E_{LUMO} (Energy of Lowest Occupied Molecular Orbital), ionization potential (IP) and electron affinity of the selected CDs were calculated using a BioMed Cache 6.1.10: (Fujitsu Ltd, Tokyo, Japan), a computer-aided molecular design modeling tool for Windows ME and XP operating systems. The structures of the selected CDs were built in the workplace and the sample files were subjected to energy minimization using augmented molecular mechanics (MM3). The energy minimization continued until the energy change was less than 0.001 kcal/mol, or molecule until the molecule has been updated 300 times. All the quantum chemical descriptors were calculated by the project leader. The log P value was calculated using the atom typing scheme. E_{HOMO} and E_{LUMO} were determined after optimizing the molecular geometry first using augmented MM2 and then using MOPAC with PM3 parameters. The ionization potential and electron affinity were approximated from the energy of the HOMO and LUMO respectively, after optimizing the molecular geometry using augmented MM2 followed by MOPAC with PM3 parameter. Dipole moment (DM) was calculated by MOPAC using AM1 at minimum energy geometry. The minimum energy geometry was obtained first optimizing with augmented MM2 and then with MOPAC using AM1 parameters. The solvent-accessible surface area (SASA) was calculated at an optimized geometry in water. The water geometry was done by optimizing and using MOPAC with PM3 parameters and the conductor-like screening model (COSMO). Absolute hardness (η) was calculated as $\eta = (E_{LUMO} - E_{HOMO})/2$.

2.5. Statistical analysis

The experimental values summarized for DPPH radical scavenging assays are expressed as the mean ± standard deviation (SD). For cytotoxicity studies the significance of the difference from the respective controls for each experimental test condition was assayed by using the Student *t* test for each paired experiment. A p value < 0.05 was considered as a significant difference when compared with control set. Linear regression analysis was used to calculate the IC_{50} values.

3. Results

3.1. Antioxidant activity and calculation of physico-chemical properties

The results of the DPPH radical scavenging activities of the CDs are summarized in Table 1. The results obtained clearly indicate the concentration-dependent activity of the selected CDs towards the DPPH radical scavenging activity. The order of reactivity was C6 (95%) > C7 (88%) > C5 (82%) > C8 (69%). The remaining CDs showed activity in the range of 13–67% at 100 μM as compared to ascorbic acid (95%). The selected quantum chemical descriptors calculated for the CDs are summarized in Table 3. The CDs described in the present investigations showed log P values in the range 0.542–3.184 (Table 3). The higher and lower HOMO energy was shown by C15 (−8.61 eV) and C2 (−9.48 eV) respectively, while higher and lower LUMO energy was calculated for C12 (−0.82 eV)

Table 1 Profile of DPPH radical scavenging activity of selected coumarin derivatives

Structure number	Concentration (μM/mL)	DPPH scavenging activity (%)	Structure number	DPPH scavenging activity (%)
C1	10	46.78 ± 0.56	C9	26.31 ± 0.13
	50	58.47 ± 0.83		28.65 ± 0.26
	100	67.83 ± 0.50		30.40 ± 0.52
C2	10	34.50 ± 0.78	C10	3.74 ± 0.09
	50	40.35 ± 1.12		11.35 ± 0.18
	100	53.81 ± 0.20		13.35 ± 0.18
C3	10	9.94 ± 0.38	C11	23.65 ± 0.74
	50	11.11 ± 0.77		29.25 ± 0.36
	100	14.61 ± 0.48		36.14 ± 0.26
C4	10	19.38 ± 0.72	C12	21.05 ± 0.30
	50	22.22 ± 1.11		26.31 ± 0.39
	100	38.01 ± 1.15		37.42 ± 0.28
C5	10	51.46 ± 0.66	C13	21.83 ± 0.09
	50	78.94 ± 0.60		24.01 ± 0.49
	100	82.45 ± 0.59		27.32 ± 0.22
C6	10	63.75 ± 0.39	C14	2.43 ± 0.11
	50	90.39 ± 0.23		6.5 ± 0.28
	100	95.63 ± 0.33		30.13 ± 0.08
C7	10	32.74 ± 0.26	C15	17.42 ± 0.31
	50	47.35 ± 0.85		19.17 ± 0.36
	100	88.30 ± 0.61		62.44 ± 0.05
C8	10	28.65 ± 0.50	Ascorbic acid	66.81 ± 0.34
	50	52.74 ± 0.18		94.32 ± 0.12
	100	69.00 ±0.27		95.98 ± 0.32

Results are expressed as the mean values from three independent experiments ± standard deviation (SD).
DPPH = 2,2,-diphenyl-1-picrylhydrazine.

and C14 (−1.15 eV) respectively. As HOMO and LUMO reflects the ionization potential (IP) and electron affinity (EA) respectively, the values of IP and EA are similar to that of respective HOMO and LUMO energies except for the negative sign. A high DM of 5.78 Debye was shown by C12, while the low DM of 3.42 was calculated for C13. The compound C15 (264.29 A^2) showed more SASA, whereas compound C2 (176.23 A^2) showed less SASA as compared to remaining CDs.

3.2. Cytotoxicity assay (MTT) for in vitro anticancer study

The profile of cytotoxicity of the CDs against selected cancer cell lines is summarized in Table 2. The CDs such as C1 (IC_{50} = 7.51 μM), C9 (IC_{50} = 7.52 μM), C7 (IC_{50} = 7.55 μM), C10, C14 (IC_{50} = 7.60 μM) and C12 (IC_{50} = 7.61μM) were found to be more cytotoxic ($p < 0.05$) against HeLa B75 cancer cells as compared to methotrexate

Table 2 Profile of cytotoxicity of selected coumarin derivatives (IC_{50} μM) against selected cancer cell lines

Structure number	Cytotoxicity (IC_{50} μM)		
	Hela-B75	HL-60	HEP-3B
C1	7.51*	9.71	10.04
C2	9.93	7.68*	17.32
C3	10.19	9.69	10.07
C4	9.69	10.05	7.56*
C5	9.95	17.37	17.39
C6	17.4	9.93	7.66*
C7	7.55*	17.35	10.00
C8	9.99	17.39	7.59*
C9	7.52*	7.60*	10.00
C10	7.60*	17.46	7.64*
C11	17.4	17.35	10.27
C12	7.61*	9.80	7.90*
C13	9.90	17.39	17.33
C14	7.60*	17.41	7.52*
C15	9.85	7.65*	7.53*
Methotrexate	5.43*	7.55*	4.33*

Results are expressed as the mean values of two parallel experiments.
*$p < 0.05$ when compared with control.

Table 3 Profile of quantum chemical descriptors studied *in silico* for the selected CDs

Sr. No.	log *P*	E_{HOMO} (eV)	E_{LUMO} (eV)	IP (eV)	EA (eV)	DM (debye)	SASA (A^2)	η
C1	0.919	-9.198	-0.949	9.198	0.949	3.582	176.826	4.124
C2	0.795	-9.488	-0.967	9.488	0.967	4.451	176.230	4.260
C3	1.535	-9.163	-1.062	9.163	1.062	4.846	176.999	4.050
C4	1.535	-9.224	-0.959	9.224	0.959	3.737	177.242	4.132
C5	1.251	-9.285	-1.009	9.285	1.009	4.583	184.444	4.138
C6	1.404	-9.251	-0.975	9.251	0.975	4.928	199.830	4.138
C7	1.567	-9.166	-0.915	9.166	0.915	3.834	196.033	4.125
C8	0.542	-9.124	-0.991	9.124	0.991	5.166	204.412	4.561
C9	1.689	-9.126	-1.029	9.126	1.029	5.267	192.568	4.048
C10	2.003	-9.181	-0.948	9.181	0.948	4.082	192.224	4.116
C11	1.404	-9.156	-0.841	9.156	0.841	4.837	198.861	4.157
C12	1.445	-9.217	-0.824	9.217	0.824	5.782	214.170	4.196
C13	2.434	-8.979	-0.853	8.979	0.853	3.429	221.495	4.063
C14	3.184	-8.774	-1.150	8.774	1.150	3.752	262.619	3.812
C15	1.721	-8.619	-1.003	8.619	1.003	3.813	264.294	3.808

(IC_{50} = 5.43 μM). The CDs such as C9 (IC_{50} = 7.60 μM), C15 (IC_{50} = 7.65 μM) and C2 (IC_{50} = 7.6 μM) showed significant cytotoxicity ($p < 0.05$) against HL 60 cancer cells as compared to methotrexate (IC_{50} = 7.55 μM). Whereas the CDs such as C14 (IC_{50} = 7.52 μM), C15 (IC_{50} = 7.53 μM), C4 (IC_{50} = 7.56 μM), C8 (IC_{50} = 7.59 μM), (IC_{50} = 7.66 μM) and C12 (IC_{50} = 7.90 μM) showed effective ($p < 0.05$) cytotoxicity against the HEP 3B cancer cells as compared to methotrexate (IC_{50} = 4.33 μM). However the compounds C9, C10, C12, C14 and C15 can be considered as lead molecules for designing novel cytotoxic agents as these molecules were found to be active cytotoxic agents ($IC_{50} < 8.0$ μM and $p < 0.05$) against most of the selected cell lines.

4. Discussion

4.1. Antioxidant activity and physico-chemical properties of selected CDs

The free radical scavenging activities of CDs are related to the number and position of the hydroxyl group on the benzenoid ring of the coumarin system. Moreover in hydroxylated coumarin, the substituent at C-2, C-4, C-7 positions is reported to play a key role in enhancing the activity.[14] Our findings are in agreement with the above described hydroxyl substitutions, as it was observed that the 4-hydroxy substituted CD (C8) and 7-hydroxy substituted CDs such as C5, C6 and C7 have demonstrated significant DPPH radical scavenging activity as compared to remaining selected CDs.

The aim of screening the selected CDs *in silico* was to evaluate the drug likelihood of these molecules and to assess their potentials to accept or donate electrons, which is a key factor for free radical reactions and for other drug-related biological activities. The selected quantum chemical descriptors calculated for the test CDs (Table 3) have a close relationship with free radical reactions and the overall biological activities of drug concern.[15]

The log P is the logarithm of the partition coefficient between *n*-octanol and water. It is an important property of drug solubility and most widely referred as an index of lipophilicity and a measure of the ability of a drug molecule to cross biological membranes. As suggested by Lipinski's rule of five, log P values more than 5 and less than −1 may not qualify a drug candidate and such compounds might not be appropriate for *in vivo* administration.[16] The HOMO and LUMO orbital energies are closely associated with the free radical scavenging activities of the antioxidant molecules.[17] The energy of the HOMO is directly related to the ionization potential

and indicates the susceptibility of the molecule to attack by electrophiles. However, the energy of the LUMO is attributed to the electron affinity and signifies the susceptibility of the molecule toward attack by nucleophiles.[18]

Conceptually, the nucleophiles and electrophiles have close attributes with radical scavenging activities manifested under the relative energy influence of the HOMO/LUMO orbitals. Nucleophiles (electron donors) and electrophiles (electron acceptors) have a high-energy HOMO and low-energy LUMO respectively. Electron-donating atoms possess high HOMO with a loose holding of valence electron, thereby being susceptible to oxidation.[19] Substances with low ionization energy give up electrons easily and hence are likely to participate in chemical reactions. In the present studies HOMO/LUMO energy profiles of the most promising DPPH radical scavenging CDs such as C6, C7, C5 and C8 do not show higher HOMO as compared to other CDs. However, it is interesting to note that these compounds have more or less equal HOMO energy (−9.1 or −9.2 eV). Classically, the dipole moment (DM) of a substance indicates its polarity. It has been described that the solubility of a drug substance in water increases with an increase in DM and SASA.[20] With few exceptions, the DM and the SASA calculated for the active CDs like C6, C7, C8 and C5, to a greater extent are in agreement with the DPPH radical scavenging activity. While describing the mechanism of free radical scavenging activities of the CDs, it has been reported that the coumarins possessing hydroxyl groups directly recombine with free radicals and interrupt the initiation and/or propagation of the induced chain reactions.[21] As a result of the phenolic behaviour of the CDs,[22] they are also reported to act as potent metal chelators and free radical scavengers, thereby showing a powerful antioxidant effect. To show antioxidant activity, a coumarin derivative has to possess at least one hydroxyl group.[23]

4.2. Cytotoxicity assay for in vitro anticancer study

The results of the cytotoxicity (Table 2) show that the selected CDs have different responses of cytotoxicity against the selected cancer cell lines. While describing the possible mechanism of biological activities by coumarins in general it has been reported that the substituents at C-2, C-4 or C-7 of the heterocyclic ring of coumarin induce biological activities, and they are known to induce apoptosis in human leukemia cells by increasing.

cytochrome C and activating the cysteine protease 32 kDa proenzyme.[24,25] The results of the cytotoxicity studies against human tumor cells conducted using 15 different hydroxylated CDs led to the identification of a 6,7-dihydroxycoumarin derivative as a lead molecule with tumor cell-specific cytotoxicity.[14] It is also suggested that the proper substitution at the 3 and/or 4 positions of the coumarin molecule is essential for designing effective cytotoxic agents.[26] In the present studies, in general the hydroxyl substitutions in the coumarin molecule at C-3 (C1), C-4 (C8, C12, C15), and C-7 (C4, C6, C10, C14, C15) have demonstrated considerable cytotoxicity against the selected cancer cells. Various physico-chemical descriptors have been described to be related with cytotoxic activities of the CDs. Earlier studies have reported that hardness (η) and softness, other than the electron-accepting and -donating properties are important factors in estimating the cytotoxic activity of coumarin derivatives.[27,28] Although it seems difficult to establish the structure−cytotoxicity relationship in the present study, the values of hardness calculated for selected CDs can be attributed with cytotoxicity. With few exceptions (C3, C12, C13) the more promising cytotoxic compounds (C9, C10, C14 and C15) showed lower η (< 4.11) values as compared to the CDs showing relatively lower cytotoxicity. These findings are in agreement with the earlier studies.[13] The CDs like C9, C10, C12, C14 and C15 can be considered as lead molecules for designing novel cytotoxic agents as these molecules were found to be effective ($IC_{50} < 8.0$ μM and $p < 0.05$) against most selected cell lines.

In conclusion, the present studies clearly show the importance of selected CDs as an antioxidant and anticancer agents. The DPPH radical scavenging studies supplemented with *in silico* analysis highlight the significance of CDs like C6, C7, C5 and C8 as lead scaffolds for the design and development of novel antioxidant CDs. Nevertheless the CDs such as C9, C10, C12, C14 and C15 can be considered as lead molecules for maneuvering novel cytotoxic agents.

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