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ORIGINAL ARTICLE

Structure–activity relationship studies of 4-methylcoumarin derivatives as anticancer agents

Ramin Miri[1], Maryam Nejati[1], Luciano Saso[2], Fatemeh Khakdan[1], Badri Parshad[3], Divya Mathur[3], Virinder S. Parmar[3,4], Marc E. Bracke[4], Ashok K. Prasad[3], Sunil K. Sharma[3], and Omidreza Firuzi[1]

[1]Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, [2]Department of Physiology and Pharmacology "Vittorio Erspamer", Sapienza University of Rome, Rome, Italy, [3]Bioorganic Laboratory, Department of Chemistry, University of Delhi, Delhi, India, and [4]Laboratory of Experimental Cancer Research, University Hospital, University of Gent, Gent, Belgium

Abstract

Context: Cancer is a leading cause of death worldwide and novel chemotherapeutic agents with better efficacy and safety profiles are much needed. Coumarins are natural polyphenolic compounds with important pharmacological activities, which are present in many dietary plants and herbal remedies.

Objectives: The objective of this study is to investigate natural and synthetic coumarin derivatives with considerable anticancer capacity against three human cancer cell lines.

Materials and methods: We synthesized 27 coumarin derivatives (mostly having 4-methyl moiety) and examined their cytotoxic effect on three human cancer cell lines, K562 (chronic myelogenous leukemia), LS180 (colon adenocarcinoma), and MCF-7 (breast adenocarcinoma) by MTT reduction assay. Screened compounds included 7-hydroxy-4-methylcoumarins (7-HMCs), 7-acetoxy-4-methylcoumarins (7-AMCs), and different dihydroxy-4-methylcoumarin (DHMC) and diacetoxy-4-methylcoumarin (DAMC) derivatives. Some compounds with methoxy, amine, and bromine substitutions were also examined.

Results: 7,8-DHMCs bearing alkyl groups at C3 position were the most effective subgroup, and of which, the most potent is compound **11**, with an *n*-decyl chain at C3, which had IC_{50} values of 42.4, 25.2, and 25.1 μM against K562, LS180, and MCF-7 cells, respectively. The second most active subgroup was 7,8-DAMCs containing ethoxycarbonylmethyl and ethoxycarbonylethyl moieties at C3 position. Compound **27** (6-bromo-4-bromomethyl-7-hydroxycoumarin), the only derivative containing bromine also showed reasonable cytotoxic activities (IC_{50} range: 32.7–45.8 μM).

Discussion and conclusion: This structure–activity relationship (SAR) study of 4-methylcoumarins shows that further investigation of these derivatives may lead to the discovery of novel anticancer agents.

Keywords

Cancer, cell, cytotoxic, MTT, methylcoumarins

History

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Introduction

Cancer is a main cause of death around the world. It has caused 7.6 million deaths in 2008 (13% of all mortalities) and the estimates for 2030 are around 13.1 million (WHO, 2013). Available chemotherapeutic agents have several drawbacks including limited efficacy or high rate of adverse effects. Therefore, searching for new anticancer drugs is of utmost importance and could help to lower the health burden of this devastating disease.

Coumarins possess a structure of fused benzene and α-pyrone ring and belong to the large subclass of benzopyrones that are widely found in several dietary plants. They are present at high levels in cinnamon and many fruits and vegetables (Krieger et al., 2013; Ribeiro da Silva et al., 2014). Different derivatives of these compounds possess a wide range of biological activities (Venugopala et al., 2013) such as antioxidant (Morabito et al., 2010; Natella et al., 2010; Pedersen et al., 2007), anti-inflammatory (Togna et al., 2014), antimicrobial (Gupta et al., 2012), and *in vitro* (Macakova et al., 2012) and *in vivo* platelet anti-aggregation effects (Kathuria et al., 2012).

Plant-derived compounds have always provided an invaluable source for the discovery of novel anticancer agents (Cufi et al., 2013; Jassbi et al., 2014). Natural coumarins, such as osthole (Zhang et al., 2012), esculetin (Lee et al., 2013), and umbelliferone (Weber et al., 1998) as well as several coumarin containing plant extracts, have been reported to inhibit the growth of cancer cells (Li et al., 2013; Ngo et al., 2010).

We have previously synthesized several coumarin analogues and have studied their biological potential (Goel et al., 2007, 2009; Verma et al., 2011). Among the various studied derivatives, 4-methylcoumarins were found to be

Correspondence: Omidreza Firuzi, Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Sciences, 1 Neshat Street, 71348-53734 Shiraz, Iran. Tel: +98 711 230 3872. Fax: +98 711 233 2225. E-mail: firuzio@sums.ac.ir

of the highest interest. An advantageous property of 4-methylcoumarins is that due to the presence of a methyl group at C4 position, they are less likely to be metabolized to the mutagenic derivative 3,4-coumarin epoxide, by the action of liver cytochrome P450 enzymes (Vassallo et al., 2004). Furthermore, 4-methylcoumarins are assumed to lack the anticoagulant effect of warfarin derivatives, in which, the presence of the OH moiety at C4 is an important feature for efficacy (Au & Rettie, 2008; Manolov et al., 2006).

Cytotoxic effects of 4-methylcoumarin derivatives have been evaluated against cancer cells (Goel et al., 2007, 2009; Riveiro et al., 2008; Verma et al., 2011). In particular, we have previously reported that 7,8-dihydroxy-4-methylcoumarin (7,8-DHMC, **8**) induces apoptosis in cancer cells (Goel et al., 2007; Riveiro et al., 2008). Furthermore, we have also observed that 7,8-diacetoxy-4-methylcoumarin (7,8-DAMC, **14)** and 7,8-diacetoxy-4-methylthiocoumarin possess *in vitro* anticancer effect (Goel et al., 2009). However, extensive studies on the structure–cytotoxic activity of 4-methylcoumarins have not been performed yet.

In this study, we screened the anticancer potential of synthesized 4-methylcoumarins with a major focus on 7,8-DHMCs and 7,8-DAMCs derivatives against different solid tumors and hematological malignancy cells and established a structure–activity relationship (SAR) for these compounds.

Materials and methods

Reagents and chemicals

Fetal bovine serum (FBS), phosphate buffered saline (PBS), RPMI 1640, and trypsin were purchased from Biosera (Ringmer, UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), umbelliferone and penicillin/streptomycin were obtained from Sigma-Aldrich (St. Louis, MO) and Invitrogen (San Diego, CA), respectively. Cisplatin and doxorubicin were obtained from EBEWE Pharma (Unterach, Austria) and dimethyl sulfoxide was purchased from Merck (Darmstadt, Germany).

Chemistry

The C3 alkyl coumarins **1–3** and **9–11** were synthesized via Pechmann condensation of 2-alkyl ethyl acetoacetate with resorcinol/pyrogallol (Kathuria et al., 2009; Pechmann & Duisberg, 1883). The 2-alkyl ethyl acetoacetate derivatives in turn were obtained by alkylation of ethyl acetoacetate using alkyl bromide and sodium hydride (Kathuria et al., 2009). Compound **8** was synthesized by condensation of pyrogallol and ethylacetoacetate in acidic conditions (Raj et al., 1996). Compounds **4**, **12**, and **18** were synthesized by the reaction of 2-acetylsuccinate with resorcinol, pyrogallol, and phloroglucinol, respectively, under Pechmann conditions (Chakravarti, 1935; Parmar et al., 1996). In a similar manner, the compounds **5**, **13**, and **19** were obtained by reaction of diethyl 2-acetylpentanedioate with resorcinol, pyrogallol, and phloroglucinol, respectively (Li et al., 2012; Parmar et al., 1987, 1996). The hydroxycoumarins **4**, **5**, **8**, **12**, **13**, and **18** on acetylation with acetic anhydride/pyridine yield the corresponding acetylated derivatives **6**, **7**, **14**, **15**, **16**, and **20**, respectively (Parmar et al., 1987, 1988, 1996; Raj et al., 1996). Compound **17** was synthesized by methylation of compound **12** using dimethyl sulfate and anhydrous potassium carbonate (Raj et al., 1996). Compound **21** was isolated according to the reported literature procedure (Sanchez-Recillas et al., 2014). Compounds **22** and **23** were obtained by the reaction of 1,2,4-trihydroxybenzene with diethyl 2-acetylsuccinate and diethyl 2-acetylpentanedioate, respectively, followed by acetylation (Parmar et al., 1987). Compound **24** was synthesized according to the synthetic protocol established by our group (Aggarwal et al., 2012). Compound **25** was synthesized by following the reported multistep procedure and was then acetylated to yield compound **26** (Reszka et al., 2010). To synthesize compound **27**, commercially available 4-bromoresorcinol was reacted with ethyl-4-bromoacetoacetate via the literature procedure (Bourbon et al., 2012). The compound **27** is novel and the data are reported for the first time (Table 1). The structures of all the synthesized compounds in this study were unambiguously established on the basis of comparing their melting point and NMR spectra with the reported literature.

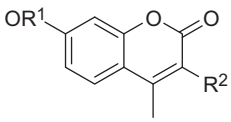
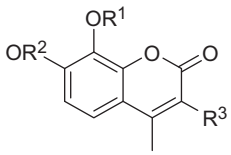
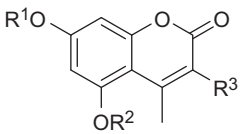
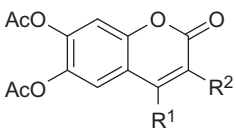
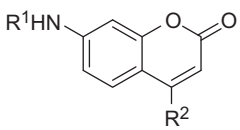
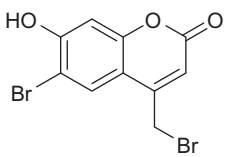
Cell lines

K562 (human chronic myelogenous leukemia), LS180 (human colon adenocarcinoma), and MCF-7 (human breast adenocarcinoma) cells were obtained from the National Cell Bank of Iran, Pasteur Institute, Tehran, Iran. These cell lines represent some of the most common solid tumors and hematological malignancies and have been used by several authors for cytotoxicity assessments (Ash et al., 2014; Liu et al., 2013; Ma et al., 2014). Cells were maintained at 37 °C in humidified air containing 5% CO_2 and were grown in monolayer cultures. All cell lines were maintained in RPMI 1640 supplemented with 10% FBS, and 100 units/mL penicillin-G and 100 μg/mL streptomycin.

Cytotoxicity assay

Cell viability following exposure to synthetic compounds was estimated by using the MTT reduction assay (Firuzi et al., 2013; Mosmann, 1983). K562, LS180, and MCF-7 cells were plated in 96-well microplates at densities of 40 000, 50 000, and 30 000cells/mL, respectively (100 μL per well). The optimal densities were determined for each cell line based on their growth curves and attention was paid to test the cells in their exponential growth phase. After overnight incubation at 37 °C, 3–4 different concentrations (ranging from 10 to 200 μM) of test compounds were added to wells in triplicate. All the compounds were first dissolved in DMSO and then diluted in the growth medium. The concentration of DMSO in the wells did not exceed 0.5%. Cells were incubated further for 72 h and then the medium was replaced with the RPMI medium without phenol red containing 0.5 mg/mL of MTT. Plates were incubated for another 4 h at 37 °C, the media were removed and formazan crystals formed in the cells were dissolved in 200 μL of DMSO. The optical density was measured at 570 nm with background correction at 655 nm using a Bio-Rad microplate reader (Model 680, Bio-Rad, Hercules, CA). The percent inhibition of viability for each

Table 1. Structures of cytotoxic coumarin derivatives screened against cancer cell lines.

Structures		Substitutions			References
		R^1	**R^2**		
	**1**	H	CH_2CH_3		Kathuria et al. (2009)
	2	H	n-C_6H_{13}		Kathuria et al. (2009)
	3	H	n-$C_{10}H_{21}$		Kathuria et al. (2009)
	4	H	CH_2CO_2Et		Parmar et al. (1996)
	5	H	$CH_2CH_2CO_2Et$		Li et al. (2012)
	6	Ac	CH_2CO_2Et		Raj et al. (1996)
	7	Ac	$CH_2CH_2CO_2Et$		Parmar et al. (1988)
		R^1	**R^2**	**R^3**	
	**8**	H	H	H	Parmar et al. (1996)
	9	H	H	C_2H_5	Kathuria et al. (2009)
	1	H	H	n-C_6H_{13}	Kathuria et al. (2009)
	11	H	H	n-$C_{10}H_{21}$	Kathuria et al. (2009)
	12	H	H	CH_2CO_2Et	Chakravarti (1935)
	13	H	H	$CH_2CH_2CO_2Et$	Parmar et al. (1987)
	14	Ac	Ac	H	Parmar et al. (1996)
	15	Ac	Ac	CH_2CO_2Et	Parmar et al. (1988)
	16	Ac	Ac	$CH_2CH_2CO_2Et$	Parmar et al. (1987)
	17	Me	Me	CH_2CO_2Et	Raj et al. (1996)
		R^1	**R^2**	**R^3**	
	**18**	H	H	CH_2CO_2Et	Parmar et al. (1988)
	19	H	H	$CH_2CH_2CO_2Et$	Parmar et al. (1987)
	20	Ac	Ac	CH_2CO_2Et	Raj et al. (1996)
		R^1	**R^2**		
	**21**	H	H		Sanchez-Recillas et al. (2014)
	22	Me	CH_2CO_2Et		Parmar et al. (1987)
	23	Me	$CH_2CH_2CO_2Et$		Parmar et al. (1987)
		R^1	**R^2**		
	**24**	H	Me		Aggarwal et al. (2012)
	25	H	n-C_3H_7		Reszka et al. (2010)
	26	Ac	n-C_3H_7		Reszka et al. (2010)
	**27**				Mp: 251–252 °C. 1H NMR (400 MHz, DMSO-d6) δ 4.84 (s, 2H, CH2Br), 6.51 (s, 1H, H-3), 6.89 (s, 1H, H-8), 7.99 (s, 1H), 11.56 (br, 1H, OH)

concentration of the compound was calculated with reference to the control and IC_{50} values were calculated with the CurveExpert software version 1.34 for Windows (Sigma, Saint Louis, MO). Each experiment was repeated 3–4 times (Azizmohammadi et al., 2013; Miri et al., 2012).

Results and discussion

In this study, cytotoxic effects of 27 coumarin derivatives (Table 1) were studied against three human cancer cell lines, i.e., K562 (human chronic myelogenous leukemia), LS180 (human colon adenocarcinoma), and MCF-7 (human breast adenocarcinoma) cells (Table 2) and SAR study of the test compounds was examined.

In general, 4-methylcoumarin derivatives had similar effects on three types of cancer cells. 7,8-DHMCs, **8**–**13**, were observed to be the most active compounds. In particular, compounds **9**–**11** exhibited IC_{50} values of less than 100 μM in all the cell lines studied, and among these, compound **11** was found to be the most potent derivative. This derivative had the lowest IC_{50} values against LS180 and MCF-7 cell lines and the second lowest IC_{50} value against K562 cells (after compound **10**). Thus, the presence of two hydroxyl groups at C7 and C8 positions seem to improve the potency of methylcoumarins as cytotoxic agents. Derivatives with IC_{50} values of higher than 200 μM were considered inactive. Umbelliferone (7-hydroxycoumarin), a natural coumarin compound, was also tested for comparison with synthesized

Table 2. Cytotoxicity of 4-methylcoumarin derivatives against different human cancer cell lines.

	IC_{50} (μM)		
Compounds	K562	LS180	MCF-7
1	111.0 ± 28.4	>200	189.8 ± 23.6
2	90.7 ± 14.2	59.4 ± 11.9	110.5 ± 1.3
3	66.7 ± 13.7	43.8 ± 5.8	66.6 ± 1.6
4	>200	>200	89.8 ± 8.3
5	>200	>200	198.6 ± 34.5
6	>200	>200	>200
7	>200	>200	>200
8	138.7 ± 75.0	>200	98.4 ± 12.2
9	51.0 ± 14.7	67.7 ± 3.0	78.2 ± 5.6
10	40.8 ± 10.6	67.1 ± 20.8	61.3 ± 7.9
11	42.4 ± 19.6	25.2 ± 1.6	25.1 ± 2.8
12	68.0 ± 8.9	175.2 ± 34.2	131.3 ± 20.0
13	62.8 ± 23.5	>200	90.4 ± 22.5
14	>200	86.4 ± 22.8	152.6 ± 11.0
15	85.3 ± 15.7	92.0 ± 18.9	88.9 ± 3.9
16	70.7 ± 8.9	137.7 ± 11.2	72.7 ± 8.8
17	>200	>200	>200
18	165.7 ± 65.5	>200	>200
19	>200	>200	139.1 ± 6.9
20	163.3 ± 59.0	>200	183.5 ± 4.1
21	114.9 ± 57.1	57.2 ± 2.4	61.9 ± 17.6
22	70.2 ± 19.9	94.7 ± 22.4	88.0 ± 7.0
23	117.1 ± 6.2	>200	92.5 ± 22.2
24	>200	>200	>200
25	>200	154.1 ± 25.0	107.8 ± 17.7
26	>200	>200	>200
27	45.8 ± 4.8	49.0 ± 1.8	32.7 ± 1.5
Umbelliferone	>200	>200	>200
Cisplatin	5.3 ± 1.8	10.0 ± 1.7	11.9 ± 1.9
Doxorubicin	0.065 ± 0.015	0.031 ± 0.007	0.053 ± 0.013

Values represent the mean ± S.E.M. of 3–5 experiments.

4-methylcoumarin derivatives and it did not show any cytotoxic effect on the tested cell lines. None of the compounds was more potent than the standard chemotherapeutic agents, cisplatin and doxorubicin (Table 2).

The finding that 7-hydroxy-4-methylcoumarins (7-HMCs) were less potent than their corresponding dihydroxy analogues, i.e., 7,8-DHMC (e.g., compound **1** versus compound **9**, etc.) has also been observed previously for the antioxidant capacity of these compounds by our research groups (Kancheva et al., 2010; Pedersen et al., 2007).

It has been previously shown by us and other investigators that compound **8** induces apoptosis in human cancer cell lines of hematological malignancies, HL-60 and U-937 (Goel et al., 2007; Riveiro et al., 2008). However, this derivative was reported to be effective only at doses higher than 100 μM, which is in agreement with our present observations.

A previous SAR study that has examined the effect of coumarins on U-937 leukemic cells have shown that o-dihydroxy coumarins show selective toxicity towards cancer cells (Vázquez et al., 2012). Another report has shown that 7,8-DHMC is a potent anticancer agent that selectively targets leukemia cells (Vázquez et al., 2013).

Substitution of the hydroxyl group with an acetoxy group reduced the cytotoxic activity, as revealed by the comparison of compounds **4**, **5**, **8**, and **25** with compounds **6**, **7**, **14**, and **26**, respectively. A similar phenomenon was observed when the hydrogen was replaced with methyl moiety (**12** versus **17**). On the contrary, comparison of derivatives **12** and **13** with **15** and **16** shows an enhancement of the cytotoxic activity with the introduction of acetoxy group, although the change is not substantial.

With regard to the effect of C3 alkyl moiety on the cytotoxic activity in 7-HMCs, replacement of ethyl (**1**) with *n*-hexyl (**2**) and *n*-decyl (**3**) chains improved the activity against all three cell lines and it appeared to be dependent on the size of the alkyl chain (compound **3** with *n*-decyl chain is more active than compound **2** with *n*-hexyl chain). A similar situation was also observed in 7,8-DHMCs when compound **8** (with hydrogen at C3 position) was compared with compounds **9**, **10**, and **11** (with ethyl, *n*-hexyl, and *n*-decyl chains at C3 position, respectively). Among 7,8-DHMC derivatives, the longer the alkyl chain, the higher was the activity (compounds **11**, **10**, and **9**, in decreasing order of efficiency). This effect of the alkyl group on the cytotoxicity is presumably due to the enhanced lipophilicity of the longer alkyl chains that consequently enhances cell membrane penetration ability of the test compounds (Mannhold et al., 2008; Završnik et al., 2003).

Introduction of an ester moiety at position C3 is not helpful in increasing the cytotoxicity of either 7-HMCs (compounds **4** and **5**) or 7,8-DHMCs (compounds **12** and **13**) in comparison with an alkyl chain substitution. Thus, it appears that by a careful modification of the structure of these compounds, finding more potent anticancer agents is possible.

We had previously observed that 7,8-diacetoxy-4-methylcoumarin (compound **14**) and 7,8-diacetoxy-4-methylthiocoumarin possess cytotoxic effects (Goel et al., 2007). In the present report, we observed that 7,8-DAMCs **15** and **16**, bearing ethoxycarbonylmethyl and ethoxycarbonylethyl moieties at C3 position, respectively, have improved potency compared with the compound **14** (with an H at C3) in K562 and MCF-7 cell lines, but not in LS180 cells.

Compounds containing two hydroxyl moieties at C5 and C7 positions (compounds **18** and **19**) had lower potencies in comparison with the derivatives with hydroxyl moieties at C7 and C8 positions (compounds **8**–**13**). This can probably be explained by the strong effect that can be brought about by the presence of the catechol moiety (Foti et al., 2005).

Another interesting compound (**27**) that showed good activity in all the cell lines has bromo groups substituted at C4 and C6 positions. The rest of the compounds including those bearing amine and amide moieties at C7 position (compounds **24**, **25**, and **26**) were not very active against any of the cell lines.

Several studies have examined the mechanism of anticancer action of different coumarin derivatives and have shown that these compounds can induce apoptosis and also inhibit cell-cycle progression (Chen et al., 2012; Haghighi et al., 2014; Singh et al., 2011). Many of these reports have shown that coumarins up-regulate proapoptotic factors, while they down-regulate anti-apoptotic markers in cancer cells (Arbab et al., 2012; Rubio et al., 2014; Singh et al., 2011), mechanisms that could plausibly apply also to our studied methylcoumarin derivatives. For instance, it has been shown that dentatin, a natural coumarin, induces apoptosis in prostate cancer cells mediated by down-regulation of anti-apoptotic proteins (Bcl-2, Bcl-xL, and survivin), disruption of mitochondrial membrane potential, and also NF-κB inhibition

(Arbab et al., 2012). In another report, RKS262, a coumarin derivative, has shown to induce apoptosis by reduction of mitochondrial membrane depolarization potential, up-regulation of pro-apoptotic markers (Bid, Bad, and Bok), and down-regulation of pro-survival factors (Bcl-xl and Mcl-1) (Singh et al., 2011). Other investigators have demonstrated that 7-isopentenyloxycoumarin induces cell-cycle arrest at the G2/M stage in bladder cancer cells (Haghighi et al., 2014).

Conclusions

In this study, cytotoxic effects of 27 coumarin derivatives were tested against three human cancer cell lines and some of the derivatives showed promising anticancer activities. The screened derivatives included 7-HMC, 7,8-DHMC, 7-acetoxy-4-methylcoumarin (7-AMC), and 7,8-DAMC, among others. It was observed that 7,8-DHMC derivatives, followed by 7,8-DAMCs, were the most potent anticancer agents. Insertion of long alkyl moiety at C3 position generally improved the anticancer activity, probably due to the enhancement of lipophilicity and cell penetration ability. These SAR studies show that the investigation of different derivatives of 4-methylcoumarins may lead to the discovery of novel more efficient anticancer agents.

Declaration of interest

The authors report that they have no conflicts of interest. The authors wish to thank the financial support of the Vice chancellor for Research, Shiraz University of Medical sciences (Grant no. 5361). The financial support from Indo-German Science and technology Centre (IGSTC), Council of Scientific and Industrial Research (CSIR, Delhi), and University of Delhi is gratefully acknowledged. Virinder S. Parmar was supported by a Grant from EU under its Erasmus Mundus EMA-2 Svaagata Programme. Badri Parshad thanks CSIR, India, for the award of Junior Research Fellowship (JRF).

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