Structure – Cytotoxicity Relationships of a Series of Natural and Semi-Synthetic Simple Coumarins as Assessed in Two Human Tumour Cell Lines

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The cytotoxicity of 22 natural and semi-synthetic simple coumarins was evaluated in GLC_4, a human small cell lung carcinoma cell line, and in COLO 320, a human colorectal cancer cell line, using the microculture tetrazolium (MTT) assay. With IC_{50} values > 100 μM, following a continuous (96h) incubation, most coumarins exhibited only low cytotoxicity. Several compounds, however, displayed significant potencies. As far as the structure – cytotoxicity relationship is concerned, it is conspicuous that all the potentially active natural compounds possess at least two phenolic groups in either the 6,7- or 6,8-positions. In addition, the 5-formyl-6-hydroxy substituted semi-synthetic analogue was found to be potent, reflecting the importance of at least two polar functions for high cytotoxicity.

Introduction

Coumarins are widely distributed in the plant kingdom (Murray *et al.*, 1982) and are present in notable amounts in citrus fruits and vegetables such as celeriac, parsnip, egg plant, fennel leaves, parsley and tomatoes. They have been reported to possess a variety of biological activities, ranging from antimicrobial activity (Harborne, 1993), inhibition of the lipoxygenase and cyclooxygenase pathways (Kimura *et al.*, 1985; Hoult *et al.*, 1994), scavenging of reactive oxygen species (Paya *et al.*, 1992 and 1994), and antitumour activity (Maucher and Von Angerer, 1994) to causing skin dermatitis, liver damage and carcinogenesis (Murray *et al.*, 1982). In contrast, only little information is available on the cytotoxic potency of simple coumarins, that are considered to exert low mammalian toxicity (Egan *et al.*, 1990; Paya *et al.*, 1994). Due to the presence of coumarins in medicinally used plants and in the human diet, a detailed study on the cytotoxic effects of coumarins is of significance. In the present study the cytotoxicity of a series of naturally occurring and synthetic simple coumarins was evaluated in two human cancer cell lines using the microculture tetrazolium (MTT) assay.

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Materials and Methods

Test compounds

Scopoletin, 7-hydroxy-5,6-trimethoxycoumarin and 6,8-dihydroxy-5,7-dimethoxy-coumarin were isolated from *Pelargonium sidoides* DC. (Geraniaceae) according to Kayser and Kolodziej (1995), while the remaining natural coumarins were available as reference samples in the research group of H. K. The synthetic derivatives 5-formyl-6-hydroxy-7-methoxycoumarin, 5-bromo-6-hydroxy-7-methoxycoumarin, 8-bromo-7-methoxy-coumarin and 8-iodo-7-methoxycoumarin were kindly provided by Dr. Wickramasinghe, University of Münster. The identity of the compounds was proved by ^1H and ^{13}C NMR spectroscopy.

Cell lines

GLC_4, a human small cell lung carcinoma cell line, was derived from a pleural effusion at the Department of Internal Medicine, University Hos-

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pital Groningen, The Netherlands, while the COLO 320 cell line was isolated from a moderately undifferentiated adenocarcinoma of the human colon (Kolodziej *et al.*, 1995). Both lines are routinely cultured at the Department of Pharmaceutical Biology, Groningen. They grow in suspension culture, partly floating and partly attached, in RPMI 1640 medium with 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid] buffer, with L-glutamine (Gibco, Paisly, UK), supplemented with 10% fetal bovine serum (Gibco) plus 200 μg/ml streptomycin and 200 IU/ml penicillin (1670 IU penicillin G = 1 mg penicillin G as sodium salt) (Gibco). They were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The doubling time was 18–21 h for GLC_4 and 13–15 h for COLO 320. Cells were in the exponential phase of growth at the moment of testing. The viability of the cells used in the experiments exceeded 95% as determined with trypan blue.

MTT assay

Cytotoxicity after treatment of the tumour cells with the test compounds was determined using the microculture tetrazolium (MTT) assay (Carmichael *et al.*, 1987). This assay is based on the reduction of a soluble tetrazolium salt, by mitochondrial enzyme activity of viable tumour cells, into an insoluble coloured formazan product, which can be measured spectrophotometrically after dissolution. Under the experimental conditions in this study, the enzyme activity and the amount of formazan formed were proportional to the number of cells.

Concentrated stock solutions (200x) of the test compounds were made in DMSO (Merck, Darmstadt, Germany) and stored at -20 °C. Cisplatin (Aldrich, Milwaukee, WI, USA) was dissolved in distilled water immediately before use. The highest concentration of each compound tested was 200 μM. The small amount of DMSO present in the wells (maximal 0.5%) was proved not to affect the experiments. The cytotoxicity assay was carried out as recently described (Kolodziej *et al.*, 1995; Woerdenbag *et al.*, 1996). Briefly, tumour cells were incubated with a range of concentrations of the test compounds in microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) at 37 °C in a humidified incubator with 5% CO_2 for a culture period of 4 days. After adding a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA), the amount of formazan formed was measured spectrophotometrically at 515 nm, using an ELX 800 universal plate reader (Bio-Tek Instruments, Winooski, VT, USA). The IC_{50} value (the drug concentration causing 50% growth inhibition of the tumour cells, calculated using the curve fitting programme 'Graphpad') was used as a parameter for cytotoxicity (Woerdenbag *et al.*, 1996).

Statistics

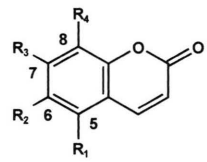
For comparison of the results of the experiments the paired Student's *t*-test was used. A p-value < 0.05 was considered significant. All cytotoxicity tests were carried out in triplicate (separate experiments).

Results and Discussion

Coumarins display wide variations in the phenolic substitution pattern of the benzopyrone nucleus, while prenylation at C-6 or C-8 quite often results in the elaboration of linear and angular furanocoumarins. With these structural modifications in mind, the cytotoxicity of a series of simple coumarins (for structures see Table I) was evaluated to obtain insight into structure-activity relationships. Cytotoxicity was examined in GLC_4, a human small cell lung carcinoma cell line, and in COLO 320, a human colorectal cancer cell line, using the microculture tetrazolium (MTT) assay. In Table II, the IC_{50} values are listed for continuous exposure (96h) of GLC_4 and COLO 320 cells to the coumarins. With IC_{50} values > 100 μM, most coumarins tested were found to be either moderately or negligibly cytotoxic, when compared with the IC_{50} value (1.0 ± 0.2 μM for GLC_4 and 2.7 ± 0.3 μM for COLO 320) of the clinically used cytostatic agent, cisplatin, which was used as a reference compound. Several compounds, however, displayed significant cytotoxicity: 6,7-dihydroxycoumarin (esculetin), 5-formyl-6-hydroxy-7-methoxy-coumarin and 6,8-dihydroxy-5,7-dimethoxy-coumarin, the latter being a metabolite from *P. sidoides* (Kayser and Kolodziej, 1995). In general, no clear differences were found regarding the cytotoxicity between the GLC_4 and COLO 320 cell lines.

Table I. Chemical structures of the coumarins used in this study.

Compound	Trivial name	R_1	R_2	R_3	R_4
Coumarin		H	H	H	H
7-Hydroxycoumarin	umbelliferone	H	H	OH	H
7-Methoxycoumarin	herniarin	H	H	OCH_3	H
6-Hydroxy-7-methoxycoumarin	scopoletin	H	OH	OCH_3	H
6,7-Dihydroxycoumarin	esculetin	H	OH	OH	H
6,7-Dimethoxycoumarin		H	OCH_3	OCH_3	H
5,6,7-Trimethoxycoumarin		OCH_3	OCH_3	OCH_3	H
7-Hydroxy-5,6-dimethoxycoumarin	umckalin	OCH_3	OCH_3	OH	H
5-Formyl-6-hydroxy-7-methoxycoumarin		CHO	OH	OCH_3	H
8-Iodo-7-methoxycoumarin		H	H	OCH_3	I
8-Bromo-7-methoxycoumarin		H	H	OCH_3	Br
8-Hydroxy-7-methoxycoumarin		H	H	OCH_3	OH
6,7-Dimethoxy-8-hydroxycoumarin	tomentin	H	OCH_3	OCH_3	OH
7,8-Dimethoxycoumarin		H	H	OCH_3	OCH_3
7-Hydroxy-8-methoxycoumarin		H	H	OH	OCH_3
5,7-Dimethoxycoumarin		OCH_3	H	OCH_3	H
5,7-Dihydroxycoumarin		OH	H	OH	H
5-Bromo-6,7-dimethoxycoumarin		Br	OCH_3	OCH_3	H
5-Hydroxy-6,7-dimethoxycoumarin		OH	OCH_3	OCH_3	H
8-Hydroxycoumarin		H	H	H	OH
6-Hydroxy-5,7-dimethoxycoumarin		OCH_3	OH	OCH_3	H
6,8-Dihydroxy-5,7-dimethoxycoumarin		OCH_3	OH	OCH_3	OH



With reference to the structure-activity relationships, it is notable that one of the active or potentially active compounds, esculetin, possesses *ortho*-dihydroxy functions, characteristic of catechol elements. Substitution of the 7-OH group by a methoxy function resulted in a significant less active compound (scopoletin). This observation confirms the recent findings of Paya *et al.* (1994) regarding the presence of catecholic functions as structural requirement for marked cytotoxic effects. However, it should also be noted that replacement of the 6,7-dihydroxy functions of esculetin by dimethoxy groups increased the cytotoxic potency, when compared with 6-hydroxy-7-methoxycoumarin (scopoletin), thereby emphasizing the difficulty in predicting cytotoxicity for oxygenated simple coumarins. Independent support for such conjecture is provided by comparing the cytotoxic effects of 8-hydroxy-7-methoxycoumarin and its 7-hydroxy-8-methoxy analogue (Table II). That derivatives with *meta*-dihydroxy arrangements may be significantly cytotoxic, as evidenced by the IC_{50} for 6,8-dihydroxy-5,7-dimethoxycoumarin (Table II), is unique and cannot satisfactorily be explained at the present but by the overall highly oxygenation pattern. The relatively high potency of the remaining active coumarin, 5-formyl-6-hydroxy-7-methoxycoumarin, should be caused by the well-known reactivity of the formyl functionality rather than by the oxygenation pattern.

In a series of 7-methoxy-8-substituted-coumarins, enhancement in cytotoxicity was observed in the following order: OCH_3 < OH < Br < I. Also, within disubstituted coumarins introduction of additional functionalities significantly reduced the IC_{50} values, as indicated by 6,7-dimethoxycoumarin *vs.* its 5-hydroxy, 5-methoxy and 8-hydroxy analogues and 5,7-dimethoxycoumarin *vs.* 6-hydroxy-5,7-dimethoxycoumarin (Table II); the highly oxygenated metabolite, 6,8-dihydroxy-5,7-dimethoxycoumarin, being an exception to this observation (*vide supra*).

Within the group of monosubstituted coumarins, 7-hydroxycoumarin (umbelliferone) was found to

Table II. Cytotoxicity of coumarins against GLC_4 and COLO 320 after continuous incubation (4 days) using the MTT assay. Given are the IC_{50} values (μM) and the growth inhibition (%GI) at 200 μM (mean ± sd; n = 3).

Compound	**GLC_4**		**COLO 320**	
	GI (%)	IC_{50}	GI (%)	IC_{50}
Coumarin	57 ± 4	190 ± 6	79 ± 4	138 ± 6
7-Hydroxycoumarin (umbelliferone)	0	>200	24 ± 5	>200
7-Methoxycoumarin (herniarin)	56 ± 5	192 ± 6	79 ± 6	147 ± 12
6-Hydroxy-7-methoxycoumarin (scopoletin)	52 ± 5	195 ± 7	82 ± 5	141 ± 11
6,7-Dihydroxycoumarin (esculetin)	100	43.5 ± 7.0	100	7.2 ± 6.7
6,7-Dimethoxycoumarin	91 ± 4	135 ± 6	89 ± 4	123 ± 4
5,6,7-Trimethoxycoumarin	54 ± 7	191 ± 18	79 ± 3	150 ± 5
7-Hydroxy-5,6-dimethoxycoumarin (umckalin)	13 ± 3	>200	3 ± 2	>200
5-Formyl-6-hydroxy-7-methoxycoumarin	100	16.4 ± 3.0	100	16.1 ± 1.9
8-Iodo-7-methoxycoumarin	88 ± 5	81 ± 10	94 ± 5	99 ± 8
8-Bromo-7-methoxycoumarin	67 ± 6	116 ± 7	79 ± 7	140 ± 8
8-Hydroxy-7-methoxycoumarin	61 ± 3	179 ± 5	84 ± 4	145 ± 4
8-Hydroxy-6,7-dimethoxycoumarin (tomentin)	70 ± 4	160 ± 9	81 ± 3	152 ± 7
7,8-Dimethoxycoumarin	52 ± 8	195 ± 17	71 ± 3	169 ± 7
7-Hydroxy-8-methoxycoumarin	68 ± 6	164 ± 11	83 ± 4	144 ± 4
5,7-Dimethoxycoumarin	68 ± 4	159 ± 6	82 ± 5	144 ± 18
5,7-Dihydroxycoumarin	71 ± 5	146 ± 6	85 ± 6	138 ± 7
5-Bromo-6,7-dimethoxycoumarin	68 ± 2	119 ± 12	76 ± 7	138 ± 11
5-Hydroxy-6,7-dimethoxycoumarin	53 ± 4	193 ± 14	70 ± 4	165 ± 5
8-Hydroxycoumarin	71 ± 5	167 ± 5	83 ± 4	151 ± 4
6-Hydroxy-5,7-dimethoxycoumarin	51 ± 3	196 ± 8	81 ± 5	162 ± 9
6,8-Dihydroxy-5,7-dimethoxycoumarin	100	22.1 ± 5.0	100	9.5 ± 4.1
Cisplatin		1.0 ± 0.2		2.7 ± 0.3

be less toxic than coumarin, the simplest member of this class of compounds, whereas introduction of a hydroxy function at C-8 in the coumarin framework increased cytotoxicity compared with its 7-hydroxy analogue.

The present study reports for the first time structure-activity relationships of a series of simple coumarins regarding cytotoxicity to human tumour cell lines, GLC_4 and COLO 320. Taken together, these results show that most coumarins possess only moderate cytotoxicity to the cell lines used and that cytotoxic effects strongly depend on the nature and position of substituents. However, investigations of the structure-activity relationships of the coumarin series show that potentially cytotoxic properties are displayed only by derivatives with at least two polar functions, as reflected by the 6,7- and 6,8-dihydroxy arrangement for natural compounds and the 5-formyl-6-hydroxy substitution for a semi-synthetic analogue of similar high potency (Table II).

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