Note

Neuroprotective and Antioxidant Activities of 4-Methylcoumarins: Development of Structure–Activity Relationships

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Coumarins are a major class of polyphenols that are abundantly present in many dietary plants and possess different biological activities. Neuroprotective effect of 28 variously substituted 4-methylcoumarins was evaluated in a cell model of oxidative stress-induced neurodegeneration, which measures viability in PC12 cells challenged with hydrogen peroxide by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The inhibitory activity of these compounds against intracellular reactive oxygen species (ROS) formation was also determined by 2′,7′-dichlorofluorescein diacetate method in the same cells. Chemical redox-based assays including 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) tests were employed to explore structure–antioxidant activity relationships in a cell-free environment. The results demonstrated that 4-methylcoumarins containing *ortho*-dihydroxy or *ortho*-diacetoxy substituents on the benzenoid ring possess considerable neuroprotective effects. *ortho*-Dihydroxy compounds inhibited cytotoxicity (44.7–62.9%) and ROS formation (41.6–71.1%) at 50 μM and showed considerable antioxidant effects. We conclude that 4-methylcoumarins are promising neuroprotective and antioxidant scaffolds potentially usefull for management of neurodegenerative diseases.

Key words 4-methylcoumarin; neuroprotective; PC12 cell; neurodegenerative disease

Coumarin derivatives containing benzopyran-2-one central core comprise a large class of plant-derived phenolic compounds that have diverse pharmacological effects and are considered as promising scaffolds for drug discovery.[1–4] Among these compounds, 4-methylcoumarins have demonstrated potential antioxidant activity with less toxicity compared to other coumarin derivatives.[5,6] Antioxidant activities of various 4-methylcoumarin derivatives have been studied by our groups and other investigators[7–11] among which, 7,8-*ortho*-dihydroxy and 7,8-*ortho*-diacetoxy structures have shown promising effects in various test systems.[8,9,12,13] Since antioxidant agents may have great potential as neuroprotective compounds, in this study, we focused on the examination of the neuroprotective effect of previously synthesized 4-methylcoumarin derivatives in oxidative stress-induced neurotoxicity model in PC12 cells with a special focus on derivatives bearing 7,8-*ortho*-dihydroxy and 7,8-*ortho*-diacetoxy substitutions.

MATERIALS AND METHODS

Chemicals RPMI1640, penicillin/streptomycin, sterile phosphate buffered saline (PBS) and trypsin ethylenediaminetetraacetic acid (EDTA) (0.25%) were purchased from Biosera (Ringmer, U.K.). Fetal bovine serum (FBS) and horse serum (HS) were acquired from Invitrogen (San Diego, CA, U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), hydrogen peroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich (San Louis, MO, U.S.A.). The rest of compounds were purchased from Merck (Darmstadt, Germany). The synthesis of 4-methylcoumarin derivatives MC1–22, MC24, MC26–28,[4] and MC23, MC25, MC29[10,11,14] containing different substituents on the benzenoid ring, were previously reported and they were synthesized according to the reported methods.

Cell Culture PC12 cells (a generous gift of Professor Lloyd A. Greene, Department of Pathology and Cell Biology, Columbia University, New York, U.S.A.) were cultured in RPMI1640 supplemented with 10% HS, 5% FBS, 100 U/mL penicillin-G and 100 μg/mL streptomycin and grown at 37°C in humidified air containing 5% CO_2.

Cell Viability Assay Cell viability after exposure to hydrogen peroxide was determined by MTT reduction assay.[15] Briefly, PC12 cells were initially plated in collagen-coated 96-well microplates at a density of 5×10^5 cells/mL (100 μL per well). After 48 h incubation, derivatives were added for 3 h, followed by H_2O_2 150 μM for 1 h. After 24 h, the cells were incubated in 0.5 mg/mL MTT dissolved in RPMI1640 for 1.5 h at 37°C. The generated formazan crystals were dissolved in 200 μL dimethyl sulfoxide (DMSO) and absorbance was measured at 570 nm. Experiments were performed in triplicate and were repeated 4–6 times. Viability was calculated as percentage compared to non-treated control cells. NAC, a known antioxidant and neuroprotective agent,[16] was used as a positive control.

Measurement of Intracellular Reactive Oxygen Species (ROS) by DCFH-DA Intracellular accumulation of ROS

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was monitored using the fluorescent probe DCFH-DA.[17] PC12 cells (4×10^5 cells/mL) were seeded in collagen-coated 24-well plates (500 μL per well) for 72 h. Test compounds were added for 3 h, followed by DCFH-DA 10 μM for 30 min at 37°C in the dark, afterwards, the media was replaced with PBS. Fluorescence intensity was quantified with a multi-mode microplate reader (BMG Labtech, Germany) at 485 and 520 nm excitation and emission filters (e_0), respectively. After 1 h of treatment with H_2O_2 150 μM, fluorescence was measured again (e_{60}). ΔF was calculated as the differences between e_0 and e_{60}. ROS inhibition was determined as follows:

$$\%\ \text{ROS Inhibition} = 100\frac{(\Delta F\ \text{Oxidized control} - \Delta F\ \text{Sample})}{(\Delta F\ \text{Oxidized control} - \Delta F\ \text{Nonoxidized control})}$$

DPPH Radical Scavenging Activity Four different concentrations of test compounds were mixed with a methanolic solution of DPPH (100 μM) and incubated at room temperature for 30 min. Absorbance at 517 nm was measured by a spectrophotometer. IC_{50} values were calculated by the software Curve-Expert (for Windows, version 1.34). Quercetin was used an standard antioxidant agent.[18]

Ferric Reducing Antioxidant Power (FRAP) Assay FRAP solution was freshly prepared by mixing 10 mL acetate buffer (300 mM) at pH 3.6, 1 mL ferric chloride (20 mM) and 1 mL 2,4,6-tris(2-pyridyl)-*s*-triazine (10 mM). Ten microliters of different concentrations of 4-methylcoumarin derivatives and quercetin dissolved in DMSO were mixed with 190 μL of FRAP solution in 96-well microplates and incubated at room temperature for 30 min. Absorbance at 595 nm was measured and FRAP values calculated.[19]

RESULTS AND DISCUSSION

Antioxidant activities of synthetic 4-methylcoumarin derivatives were assessed using different cell-based and cell-free assays. Studied 4-methylcoumarins ate categorized into five groups (Fig. 1): 1) monohydroxy 4-methylcoumarins (MHMCs) containing hydroxyl group at the C-7 position (MC1–5); 2) dihydroxy 4-methylcoumarins with two hydroxyl groups at 7,8 positions (7,8-DHMC, MC6–11) or 5,7 positions of the coumarin ring (5,7-DHMC, MC12–13); 3) monoacetoxy 4-methylcoumarins (MAMCs) having acetoxy group at the C-7 position (MC14–15); 4) diacetoxy 4-methylcoumarins (DAMCs), including 7,8-DAMC (MC16–18), 5,7-DAMC (MC19) and 6,7-DAMC (MC20–21); 5) dimethoxy 4-methylcoumarins bearing two methoxy groups at the C-7 and C-8 positions (7,8-DMMC, M23–M25); 6) miscellaneous derivatives with different substitutions.

Protective effect of 4-methylcoumarins against H_2O_2-induced cytotoxicity in PC12 cells was determined by the MTT reduction assay (Fig. 2, not-active compounds are not shown). Tested compounds' inhibitory activity against ROS formation induced by H_2O_2 in PC12 cells was also measured by DCFH-

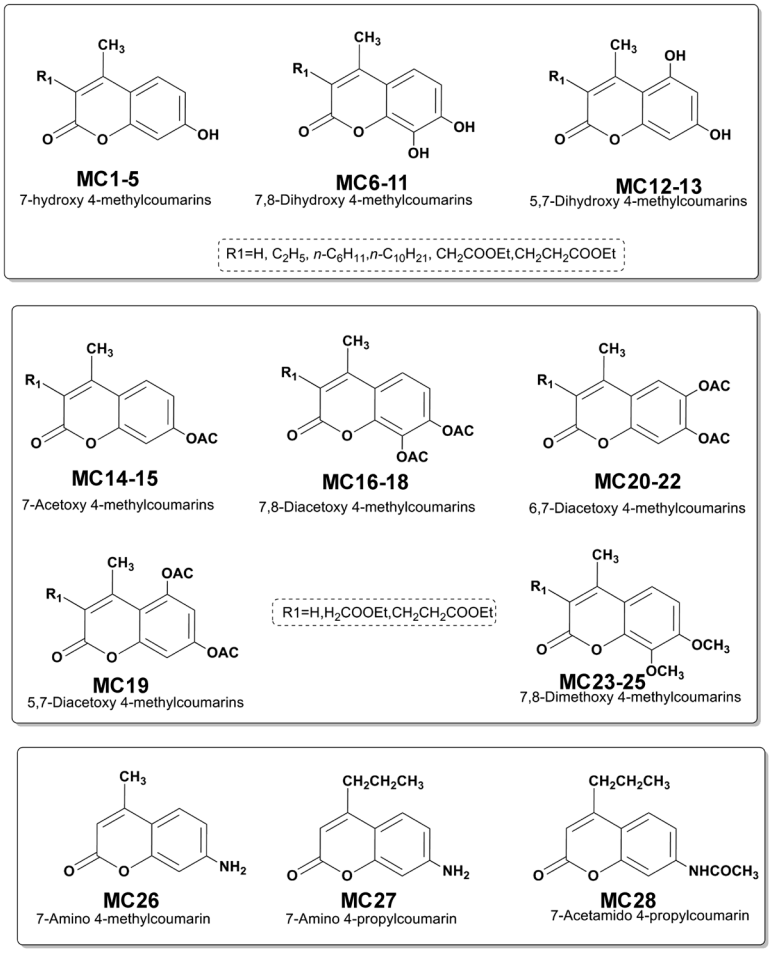


Fig. 1. General Structures of Synthesized 4-Methylcoumarins

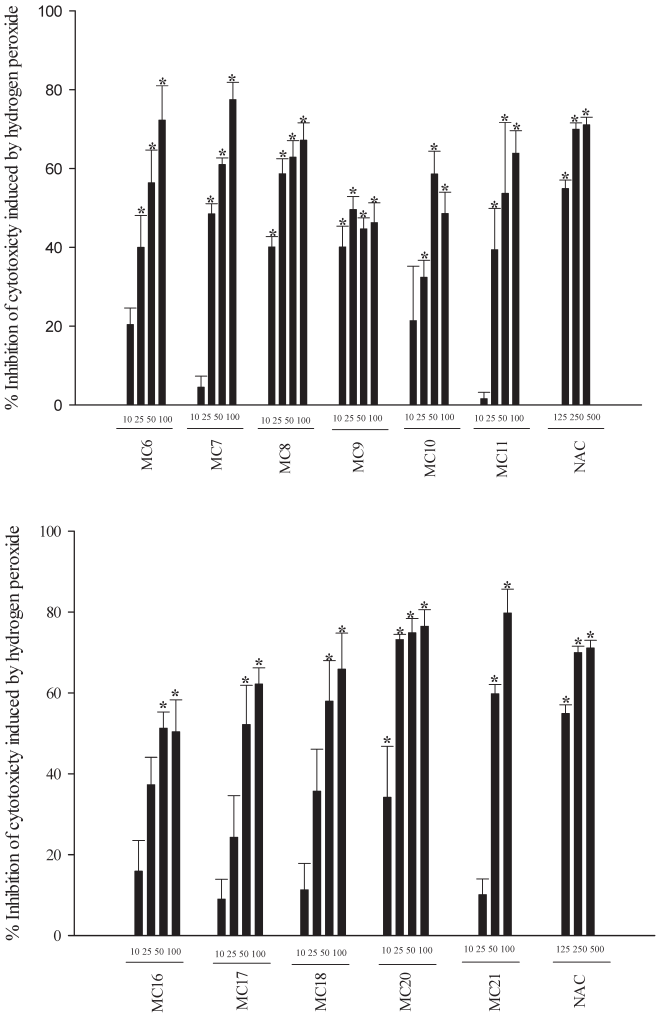
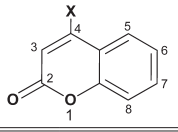


Fig. 2. Inhibition of Hydrogen Peroxide-Induced Cytotoxicity in PC12 Neuronal Cells by Synthesized 4-Methylcoumarin Derivatives

Protective effects of 7,8-dihydroxymethylcoumarines (upper panel) and 7,8-diacetoxymethylcoumarines (lower panel) against H_2O_2 was determined in PC12 cells. Concentrations of tested methylcoumarins and *N*-acetylcysteine (NAC, positive control) are expressed as μM. * Significantly different from cells treated with hydrogen peroxide alone ($p<0.05$).

Table 1. Inhibition of Hydrogen Peroxide-Induced Cytotoxicity and Intracellular ROS Formation by 4-Methylcoumarin Derivatives in PC12 Neuronal Cells



Compound	X	Substitution on benzenoid ring	Substitution at C3 position	Inhibition of neurotoxicity induced by H_2O_2* (% Protection at 50 μM)	Intracellular ROS inhibition (% Protection at 50 μM)
MC1	CH_3	7-Hydroxy	CH_2CH_3	NA**	NA
MC2	CH_3	7-Hydroxy	*n*-C_6H_{13}	NA	NA
MC3	CH_3	7-Hydroxy	*n*-$C_{10}H_{21}$	NA	NA
MC4	CH_3	7-Hydroxy	$CH_2CO_2C_2H_5$	NA	NA
MC5	CH_3	7-Hydroxy	$CH_2CH_2CO_2C_2H_5$	NA	NA
MC6	CH_3	7,8-Dihydroxy	H	56.4±8.3	41.6±13.2
MC7	CH_3	7,8-Dihydroxy	C_2H_5	61.0±1.7	71.1±12.0
MC8	CH_3	7,8-Dihydroxy	*n*-C_6H_{13}	62.9±4.2	69.1±7.5
MC9	CH_3	7,8-Dihydroxy	*n*-$C_{10}H_{21}$	44.7±2.8	46.7±13.0
MC10	CH_3	7,8-Dihydroxy	$CH_2CO_2C_2H_5$	58.6±5.8	63.5±13.4
MC11	CH_3	7,8-Dihydroxy	$CH_2CH_2CO_2C_2H_5$	53.7±18.0	46.8±8.7
MC12	CH_3	5,7-Dihydroxy	$CH_2CO_2C_2H_5$	NA	NA
MC13	CH_3	5,7-Dihydroxy	$CH_2CH_2CO_2C_2H_5$	NA	NA
MC14	CH_3	7-Acetoxy	$CH_2CO_2C_2H_5$	NA	NA
MC15	CH_3	7-Acetoxy	$CH_2CH_2CO_2C_2H_5$	NA	NA
MC16	CH_3	7,8-Diacetoxy	H	51.3±4.0	35.4±8.4
MC17	CH_3	7,8-Diacetoxy	$CH_2CO_2C_2H_5$	52.2±9.7	51.5±7.2
MC18	CH_3	7,8-Diacetoxy	$CH_2CH_2CO_2C_2H_5$	58.0±10.0	19.5±11.1
MC19	CH_3	5,7-Diacetoxy	$CH_2CO_2C_2H_5$	NA	NA
MC20	CH_3	6,7-Diacetoxy	H	74.9±3.5	37.0±14.0
MC21	CH_3	6,7-Diacetoxy	$CH_2CO_2C_2H_5$	59.8±2.3	45.5±9.1
MC22	CH_3	6,7-Diacetoxy	$CH_2CH_2CO_2C_2H_5$	NA	NA
MC23	CH_3	7,8-Dimethoxy	H	NA	NA
MC24	CH_3	7,8-Dimethoxy	$CH_2CO_2C_2H_5$	NA	NA
MC25	CH_3	7,8-Dimethoxy	$CH_2CH_2CO_2C_2H_5$	NA	NA
MC26	CH_3	7-NH_2	H	NA	NA
MC27	$CH_2CH_2CH_3$	7-NH_2	H	NA	NA
MC28	$CH_2CH_2CH_3$	7-NHAC	H	NA	NA

* Data are expressed as the mean±S.E.M. of 4–6 experiments. ** NA: Not active.

DA assay (Table 1). The *ortho* disubstituted 4-methylcoumarins, including 7,8-DHMCs (MC6 to 9), 7,8-DAMCs (MC16 to 18) and 6,7-DAMCs (MC20, and 21, but not 22) demonstrated considerable inhibitory effects against neurotoxicity and ROS formation, which were for some of the derivative comparable to the effect of NAC, a reference antioxidant agent. 4-Methylcoumarins containing two hydroxy or two acetoxy moieties at *meta* position to each other including 5,7-DHMC (MC12–13) and 5,7-DAMC (MC19) were found to be devoid of the protective effect against H_2O_2-induced neurotoxicity, ROS formation and antioxidant activity (Tables 1, 2). Moreover, substitution by methoxy, amino or acetamido moieties at the benzenoid ring also eliminated the protective effects of compounds against oxidative stress. The superior activity of 7,8-DHMCs compared to monohydroxy compounds (7-MHMCs) and 5,7-DHMCs might be attributed to the increased stability of the catecholic radical as a result of electron donating characteristic of *ortho*-dihydroxy substituents.

Although it has been repeatedly shown that *ortho*-dihydroxy moiety confers a high antioxidant potential,[13] in our study the substitution of the catechol moiety with diacetoxy (as in 7,8-DAMCs and 6,7-DAMCs) did not considerably alter the activities. This particular phenomenon in 4-methylcoumarins has been previously reported in literature[20,21] and at least in biological systems may be ascribed to the production of deacetylated compounds through the activity of esterase enzymes.[21]

Previous reports have shown that insertion of ester and other moieties at C3 position may alter the antioxidant capacities of 4-methylcoumarines,[21] therefore we examined the influence of these moieties on the activity of derivatives in our test systems; among 7,8-DHMCs, the presence of an alkyl chain such as ethyl (MC7), *n*-hexyl (MC8) and *n*-decyl (MC9) or ethoxycarbonyl methyl (MC10) and ethoxycarbonyl ethyl (MC11) moieties at C3 did not seem to considerably alter the neuroprotective, ROS inhibition or antioxidant activities in the active subset of 7,8-DHMCs. The same phenomenon was observed among the other two active subsets of 7,8-DAMCs and 6,7-DAMCs. The only exception seemed to be in the case of MC22 (a 6,7-DAMC) that the presence of an ethoxycarbonyl ethyl moiety completely abolished neuroprotective and anti-

Table 2. Antioxidant Activity of 4-Methylcoumarin Derivatives Determined by DPPH and FRAP Assays

Compound	IC_{50} DPPH scavenging (μM)	FRAP value (mM Q/mol)*
MC1	3769.5±592.9	3.9±0.4
MC2	NA**	2.2±0.3
MC3	NA	4.3±0.6
MC4	NA	7.4±0.9
MC5	NA	0.6±0.0
MC6	ND	ND
MC7	15.8±3.3	964.0±51.1
MC8	18.0±3.0	779.5±30.7
MC9	17.5±3.1	667.4±82.8
MC10	15.4±3.4	660.0±136.3
MC11	12.1±0.6	1109.4±3.5
MC12	819.0±80.7	1.4±0.3
MC13	537.1±50.8	2.3±0.5
MC14	NA	0.3±0.0
MC15	ND	ND
MC16	ND	ND
MC17	97.8±4.8	35.8±1.9
MC18	37.9±7.7	119.3±17.6
MC19	3088.1±539.7	1.0±0.2
MC20	6.5±1.1	1489.2±100.3
MC21	23.3±9.3	499.0±10.5
MC22	NA	2.5±0.4
MC23	ND	ND
MC24	NA	1.2±0.1
MC25	ND	ND
MC26	NA	0.9±0.1
MC27	NA	1.4±0.3
MC28	NA	1.0±0.2
Quercetin	7.9±0.3	1000

* mM Equivalent of quercetin/mol. ** Not active (IC_{50}>5000 μM). Values represent the mean of 3–5 experiments±S.D.

oxidant effects (Tables 1, 2). Similar to this finding, another study on galvynoxyl and DPPH radicals scavenging capacity of 4-methylcoumarins has found that the presence of ethoxycarbonyl ethyl moiety at C3 position increases the activity of *meta*- and *ortho*-dihydroxy coumarins, but not the activity of *ortho*-diacetoxy derivatives.[21]

The findings of antioxidant assays (Table 2) closely resembled those obtained in cellular assays. Some of the compounds showed activities comparable to that of quercetin, a known antioxidant agent.

In conclusion, 4-methylcoumarins containing *ortho*-dihydroxy or *ortho*-diacetoxy substituents on the benzenoid ring showed considerable potential in inhibition of oxidative stress-induced neuronal damage in a cell model of neurodegeneration and also in cell-free antioxidant assays and represent promising scaffolds with antioxidnat and neuroprotective activities.

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Conflict of Interest The authors declare no conflict of interest.

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