JPP Journal of Pharmacy and Pharmacology

JPP 2007, 59: 1721–1728

Received March 29, 2007
Accepted July 17, 2007
DOI 10.1211/jpp.59.12.0015
ISSN 0022-3573

Antioxidant activity of 4-methylcoumarins

Jens Z. Pedersen, Cristina Oliveira, Sandra Incerpi, Vineet Kumar, Anna Maria Fiore, Paolo De Vito, Ashok K. Prasad, Sanjay V. Malhotra, Virinder S. Parmar and Luciano Saso

Abstract

Polyphenolic coumarins are known to act as antioxidants in biological systems, but it is difficult to distinguish their antioxidant activity from the many other effects they produce in cells. We have determined the radical scavenging capacity of 22 structurally related natural and synthetic 4-methylcoumarins, by measuring their reaction with radicals, galvinoxyl and 2,2-diphenyl-1-picrylhydrazyl, using electron paramagnetic resonance spectroscopy. Efficient antioxidant activity of 4-methylcoumarins in cells was verified using the DCF fluorescent probe assay for determination of intracellular reactive oxygen species levels. As expected, the *o*-dihydroxysubstituted coumarins were found to be excellent radical scavengers and better than the *m*-dihydroxysubstituted or monohydroxysubstituted analogues, but surprisingly the corresponding *o*-diacetoxy derivatives also turned out to be good scavengers, even in the absence of an esterase. Another unexpected result was that the antioxidant efficiency of 4-methylcoumarins could be modulated by introducing an ethoxycarbonylethyl substituent at the C-3 position; this effect cannot be explained by simple electron donating/withdrawing properties. Coumarin concentrations of 10 μM or less were used in all experiments, corresponding to the levels relevant for therapeutic purposes. Considering that 4-methylcoumarins, in contrast to many other coumarins, are not metabolized to toxic epoxide intermediates, these results indicate promising new strategies for the design of non-toxic antioxidant coumarin-based drugs.

Department of Biology, University of Rome 'Tor Vergata', Italy

Jens Z. Pedersen, Paolo De Vito

Department of Biology, University of Rome 'Roma Tre', Italy

Sandra Incerpi, Anna Maria Fiore

Department of Chemistry, University of Delhi, India

Vineet Kumar, Ashok K. Prasad, Virinder S. Parmar

Department of Chemistry and Environmental Science, New Jersey Institute of Technology, Newark, USA

Vineet Kumar, Sanjay V. Malhotra

Department of Human Physiology and Pharmacology, Sapienza University of Rome, Italy

Luciano Saso, Cristina Oliveira

Correspondence: J. Z. Pedersen, Department of Biology, University of Rome 'Tor Vergata', Via della Ricerca Scientifica 1, 00133 Roma, Italy.
E-mail: jzp@ofir.dk

Acknowledgement: The financial support from the Italian Ministry for University and Research, General Management for Strategies and Development of Internationalization of Scientific and Technological Research is gratefully acknowledged.

Introduction

Coumarins are a group of compounds that show a surprising variety of biological effects. They occur naturally in many plants, fungi and bacteria, and have found applications for centuries as spices and in traditional medicine. Several natural polyphenolic coumarins show anti-inflammatory, antimicrobial, antiviral, anti-carcinogenic, anticoagulant and antioxidant activity; but generally the reason for these effects and the precise nature of their actions are not known (Borges et al 2005). Many different types of antioxidant activity of coumarins have been reported for a variety of biological systems, but it has not yet been possible to correlate the effects observed to the chemical structures of the coumarins studied (Fylaktakidou et al 2004; Borges et al 2005; Kostova 2006). Hydroxycoumarins are believed to behave like classic phenol- or quinol-based antioxidants, in which a hydroxy group on an aromatic ring structure can carry out the single-electron reduction of a free radical. The resulting phenoxyl radical or semiquinone can either be stabilized through the presence of bulky or electron-withdrawing groups on the ring system or be oxidized further through the consecutive single-electron reduction by a second hydroxy group to produce a quinone-type end product. This is the mechanism behind both natural and synthetic antioxidants, like vitamin E and butylated hydroxytoluene; however, sometimes it turns out that coumarins do not work in this way (Fylaktakidou et al 2004; Kostova 2006).

One problem with coumarin compounds has been the tendency to form 3,4-coumarin epoxides during metabolic degradation; these intermediates are believed to be mutagenic and probably also have other toxic effects. To prevent this problem a series of 4-methylcoumarins have been synthesized; in these compounds 3,4-coumarin epoxide formation is no longer possible as 4-methylcoumarins are not substrates for the liver P-450 monoxygenases that epoxidize coumarins lacking the C-4 methyl group. Several 4-methylcoumarins have shown promising antioxidant effects, such as inhibition of lipid peroxidation and scavenging

of superoxide anions (Raj et al 1998a, b, c; Kumar et al 2005a). We here present a study of the radical scavenging capacity of a group of natural and synthetic coumarins examined under normalized conditions. Using electron paramagnetic resonance (EPR) spectroscopy we have tested 22 structurally related 4-methylcoumarins (Figure 1, Table 1), measuring the kinetics of their reaction with the standard radicals galvinoxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH). Very low concentrations have been used for these measurements (10 μM or less), corresponding to the levels that would be considered for therapeutic purposes. The antioxidant efficiency of 4-methylcoumarins in cells was verified using a standard fluorescent assay for determination of intracellular reactive oxygen species (ROS) levels. The results showed that *ortho*-dihydroxysubstituted coumarins were more efficient radical scavengers than *meta*-dihydroxysubstituted or monohydroxysubstituted molecules, but surprisingly the corresponding *ortho*-diacetoxy derivatives also turned out to be good scavengers, even in the absence of an esterase. The antioxidant efficiency of 4-methylcoumarins could be modified by changing the type of substituent at the C-3 position. These results indicate new possible strategies for the design of non-toxic coumarin antioxidants.

Materials and Methods

Reagents

All 4-methylcoumarins and 4-methylthionocoumarins were synthesized and characterized at the Department of Chemistry of the University of Delhi as previously described (Parmar et al 1996; Raj et al 1996; Singh et al 2002; Kumar et al 2005a). Galvinoxyl, 2,2-diphenyl-1-picrylhydrazyl (DPPH)

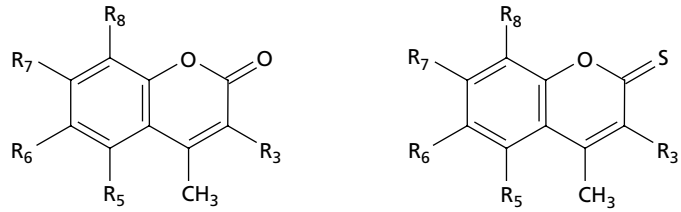

Figure 1 Structures of the coumarin derivatives studied in this work (see also Table 1).

Table 1 Structures of the 4-methylcoumarins (C1–C20) and 4-methylthionocoumarins (C21–C22) tested

Compound	R_3	R_5	R_6	R_7	R_8
C1	$CH_2COOCH_2CH_3$	OH	H	OH	H
C2	H	OH	H	OH	H
C3	$CH_2CH_2COOCH_2CH_3$	H	H	$OOCCH_3$	$OOCCH_3$
C4	$CH_2CH_2COOCH_2CH_3$	$OOCCH_3$	H	$OOCCH_3$	H
C5	H	H	H	$OOCCH_3$	$OOCCH_3$
C6	$CH_2COOCH_2CH_3$	H	H	$OOCCH_3$	$OOCCH_3$
C7	$CH_2COOCH_2CH_3$	$OOCCH_3$	H	$OOCCH_3$	H
C8	$CH_2COOCH_2CH_3$	H	H	OH	OH
C9	H	H	OH	OH	H
C10	$CH_2CH_2COOCH_2CH_3$	H	OH	OH	H
C11	$CH_2CH_2COOCH_2CH_3$	OH	H	OH	H
C12	$CH_2CH_2COOCH_2CH_3$	H	H	OCH_3	OCH_3
C13	H	OCH_3	$OOCCH_3$	OCH_3	H
C14	$CH_2CH_2COOCH_2CH_3$	H	OCH_3	OCH_3	H
C15	H	OCH_3	H	OCH_3	H
C16	$CH_2COOCH_2CH_3$	H	H	OCH_3	OCH_3
C17	$CH_2CH_2COOCH_2CH_3$	H	H	OH	OH
C18	$CH_2COOCH_2CH_3$	OCH_3	H	OCH_3	H
C19	H	H	H	OCH_3	OCH_3
C20	H	H	H	OH	OH
C21	H	H	H	$OOCCH_3$	$OOCCH_3$
C22	H	H	H	OH	OH

See also Figure 1.

and cumene hydroperoxide were purchased from Sigma-Aldrich (St Louis, MO); 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) was obtained from Molecular Probes (Eugene, OR). Dulbecco's modified Eagle's medium (DMEM), antibiotics and sterile plasticware for cell culture were from Flow Laboratory (Irvine, UK). Fetal bovine serum was from GIBCO (Grand Island, NY).

Electron paramagnetic resonance spectroscopy

Stock solutions of all 4-methylcoumarin compounds were prepared in ethanol 95% at a concentration of 5 mM. A galvinoxyl stock solution (5 mM in ethanol 95%) was freshly prepared immediately before the experiments. Systematic screening of all compounds was made with a final concentration of 10 μM in the presence of galvinoxyl at the same concentration. For some experiments, DPPH (50 μM) was used as an alternative to galvinoxyl. The solutions were drawn into glass capillaries, sealed and measured using an ESP300 instrument (Bruker Spectrospin, Karlsruhe, Germany) equipped with a high sensitivity TM_{110} X-band cavity. Radical spectra were recorded at room temperature, using 0.6 G modulation, 1 mW microwave power and a scan time of 42 s for a 30 G spectrum. Normally, four spectra were accumulated for each measurement to obtain a suitable signal-to-noise ratio. The kinetics of the reaction was followed for 3 h at room temperature or until the radical signal had disappeared.

Cell culture

L-6 cells from rat skeletal muscle were obtained from the American Type Culture Collection (Rockville, MD). Cells were seeded in 75-cm^2 flasks for tissue culture and grown in DMEM supplemented with 10% fetal bovine serum, 100 μg mL^{-1} streptomycin and 100 U mL^{-1} penicillin, in an atmosphere of 5% CO_2 at 37°C. The cells reached confluency after 5 days (about 6×10^6 cells) and were kept in culture as myoblasts by continuous passages at pre-confluent stages, as previously reported (D'Arezzo et al 2004).

Cytotoxicity assay

Toxicity of the coumarins during the experiments on intracellular ROS measurements was determined by the method of Hansen et al (1989) with some modifications. Briefly, cell viability was quantified by the conversion of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) to purple MTT formazan by the mitochondrial dehydrogenases of living cells. The experiment was carried out in six-well plates using 1×10^6 cells at the confluent state per well. Control and treated cells were incubated with MTT at a final concentration of 1 mg mL^{-1} for 3 h at 37°C. Thereafter cells were scraped off and centrifuged at 1200 rev min^{-1} for 5 min; the pellet was re-suspended in 300 μL phosphate-buffered saline (PBS) and sonicated on ice for 15 s with an Ultrasonic W-225R, at setting 4, and then centrifuged in a microfuge at 13 000 rev min^{-1} for 10 min. The supernatant was discarded and the final pellet re-suspended in 200 μL dimethyl sulfoxide (DMSO); after appropriate dilutions MTT formazan formation was measured with a spectrophotometer at 560 nm. Results are given as the mean ± standard deviation (s.d.) of 3 similar experiments carried out in triplicate.

Intracellular ROS determination

At the time of the experiment the DMEM was discarded and cells were washed twice with 5 mL PBS containing 5.0 mM glucose at 37°C. Cells were gently scraped off with 5 mL PBS plus glucose at 37°C, the cell suspension was transferred to a centrifuge tube and the buffer was added up to 8–10 mL. Cells were centrifuged at 1200 rev min^{-1} for 5 min (about 100 *g*), the supernatant was discarded and the pellet re-suspended with a plastic Pasteur pipette in 5 mL PBS. Incubation with the probe $DCFH_2$-DA at a final concentration of 10 μM (from a stock solution of 10 mM in DMSO) was carried out for 30 min in the dark at 37°C, as previously reported (Pallottini et al 2005). The cells were gently re-suspended every 10 min; at the end of incubation cells were centrifuged at 1200 rev min^{-1} for 5 min, the supernatant was discarded and the cell pellet was re-suspended in 5 mL of PBS plus glucose and centrifuged again. The final supernatant was discarded and the cell pellet re-suspended in 2 mL PBS at a final concentration of 3×10^6 cells/mL. Before starting the experiment, a recovery was carried out at room temperature for 45 min in the dark.

Intracellular fluorescence was measured under continuous gentle magnetic stirring at 37°C in a Perkin–Elmer (Norwalk, CT) LS 50B luminescence spectrometer. Excitation and emission wavelengths were set at 498 nm and 530 nm, respectively, using 5 and 10 nm slits for the two light paths. The assay was carried out in 3 mL final buffer containing 200 μL cell suspension. Cumene hydroperoxide diluted 1:100 in DMSO was used as radical generator (final concentration 300 μM); DMSO at the concentrations used did not affect the fluorescence signal. The antioxidant potency of a coumarin was determined by the decrease in the intracellular DCF fluorescence, reported as ΔF/10 min, and was calculated relative to the fluorescence change induced by 300 μM cumene hydroperoxide alone (100%). Cells were incubated with coumarins for 10 min at 37°C before the addition of cumene hydroperoxide; none of the coumarins tested gave rise to fluorescence on their own.

Statistical analysis

All assays were performed at least in triplicate and data were expressed as mean ± s.d. Statistical analyses were made using the GraphPad Prism 4.0 software; the type of test used is specified for each table and figure.

Results and Discussion

Radical scavenging activity

The 22 methylcoumarins studied here (Table 1) can be arranged into different groups according to their structure. Essentially, there are 9 dihydroxycoumarins, 7 diacetoxycoumarins and 6 dimethoxycoumarins; these groups can be subdivided further according to the positions of the substituents

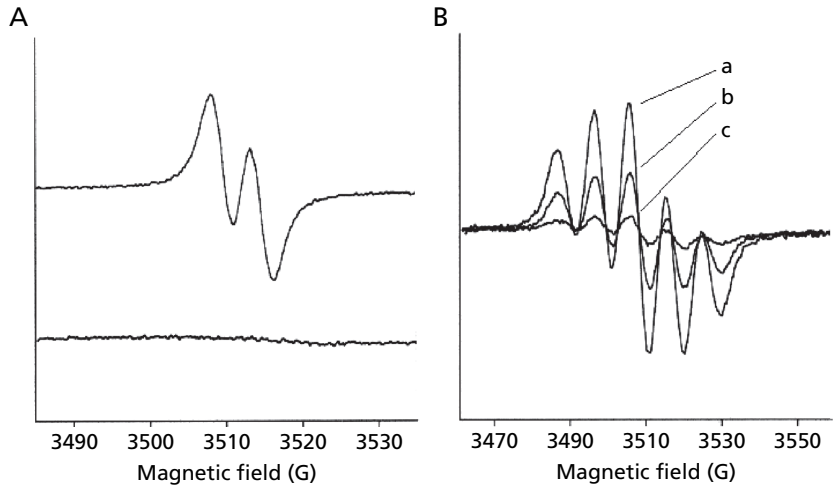

Figure 2 EPR spectra of the target radicals used. A. Spectrum of 10 μM galvinoxyl in ethanol before (top) and 1 min after addition of 10 μM C20 (bottom). B. Superimposed spectra showing the DPPH (50 μM) control (a), and the disappearance of the signal after 30 min (b) and after 120 min (c) incubation with 50 μM C1.

(7,8-*ortho*, 6,7-*ortho* or 5,7-*meta*) and the presence of an additional ester at the C-3 position. In addition, two of the compounds are thionocoumarins. The ability to scavenge free radicals was tested with a very simple assay, mixing a coumarin with a radical and following the reaction by EPR spectroscopy (Iuliano et al 1999; Shi et al 2001; McPhail et al 2003). A systematic screening of all compounds was made, with a final concentration of 10 μM in ethanol in the presence of galvinoxyl at the same concentration. Alternatively, some measurements were made with lower concentrations, or using another radical, DPPH, at a concentration of 50 μM.

The EPR spectra of galvinoxyl and DPPH are shown in Figure 2. At the concentrations used here, the height of the EPR signal is directly proportional to the concentration of the radical; when this radical is reduced by an antioxidant, the spectrum disappears. For some of the coumarins it was possible to detect the spectrum of the corresponding coumaryl radical superimposed on the galvinoxyl or DPPH spectrum immediately after mixing the sample; all the coumaryl radicals were too reactive to allow characterization and disappeared within seconds or minutes (data not shown).

All coumarins tested showed some scavenging activity, but some of them were much slower than others. The kinetics for the compounds C4, C7, C12, C13, C14, C15, C16, C18 and C19 (Figure 3) were too slow to be of interest; these are mainly dimethoxy or *m*-diacetoxy derivatives and were not expected to show significant antioxidant activity. In contrast, C3, C5, C6, C8, C9, C10, C17, C20 and C22 were so active that the galvinoxyl EPR signal disappeared immediately before the spectrum could be measured; these compounds were therefore tested at a lower coumarin concentration (1 μM), to allow detection of the radical level after 60 min (Table 2). Finally, the three *m*-dihydroxy coumarins were found to have an intermediate type activity (Figure 3).

The results obtained for galvinoxyl, a phenoxyl-type radical, were confirmed for scavenging of DPPH, a nitrogen-based radical. The same pattern of reactivity was found, with

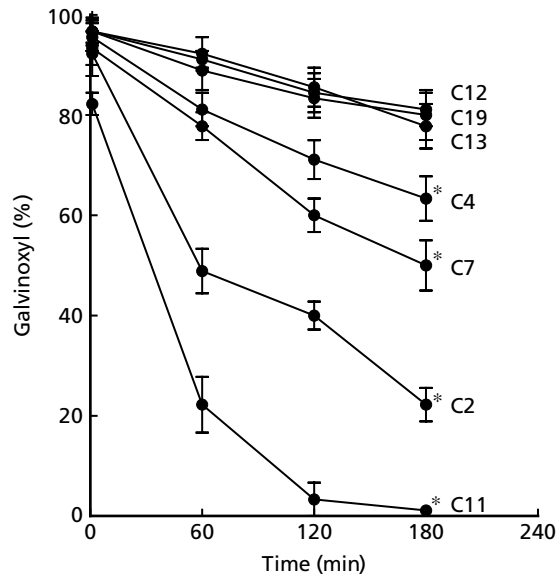

Figure 3 Kinetics of the disappearance of the galvinoxyl EPR signal due to reaction with 4-methylcoumarins. Samples contained 10 μM galvinoxyl and 10 μM coumarins in ethanol. Curves are displayed for seven different compounds. The poor scavengers, C14, C15, C16 and C18, gave curves indistinguishable from those of C12, C13 and C19. Data at 180 min were analysed using a repeated measures analysis of variance (n = 6), with individual differences evaluated with the Newman–Keuls multiple comparison test. *$P < 0.001$ compared with all other compounds.

the same three categories of very good, intermediate and poor scavengers; also, in this case, the efficient compounds were highly active at a concentration of 1 μM (Table 3). Both galvinoxyl and DPPH are strongly coloured compounds so the antioxidant reactions can also be followed spectrophotometrically; however, the products formed in the reactions are

Table 2 Effect of ethoxycarbonylmethyl or ethoxycarbonylethyl substitution at the C-3 position

4-Methylcoumarin	$R_3 = H$	$R_3 = CH_2COOCH_2CH_3$	$R_3 = CH_2CH_2COOCH_2CH_3$
7,8-Dihydroxy-[a]	25 ± 2 (C20)	23 ± 4 (C8)	11 ± 2 (C17)*
6,7-Dihydroxy-[a]	21 ± 3 (C9)	n.d.[c]	13 ± 4 (C10)*
5,7-Dihydroxy-[b]	44 ± 4 (C2)	30 ± 3 (C1)	20 ± 2 (C11)*
7,8-Diacetoxy-[a]	64 ± 3 (C5)	76 ± 5 (C6)	72 ± 5 (C3)
5,7-Diacetoxy-[b]	n.d.	70 ± 4 (C7)	73 ± 3 (C4)

Expressed as percentage of the galvinoxyl EPR signal remaining after incubation of 10 μM galvinoxyl with 4-methylcoumarins for 60 min at room temperature. Coumarin numbers are shown in brackets. Note that not all data are directly comparable, due to the different coumarin concentrations employed. *$P < 0.001$, with respect to $R_3 = H$ in the same row (Student's *t*-test). [a]Initial coumarin concentration 1 μM; [b]initial coumarin concentration 10 μM; [c]not determined, compound not available.

Table 3 Decay of the DPPH radical EPR signal during the reaction with 7,8-dihydroxy-4-methylcoumarin (C20) or with the corresponding diacetyl ester (C5)

	Incubation time = 1 min			Incubation time = 60 min		
Compound	1 μM	10 μM	50 μM	1 μM	10 μM	50 μM
C5	99 ± 2	97 ± 4	70 ± 4	96 ± 4	84 ± 3	18 ± 6
C20	95 ± 5	63 ± 1	11 ± 6	86 ± 8	43 ± 2	0

Data are shown as the percentage of radical remaining; 100% corresponds to 50 μM DPPH. Data shown are the mean ± s.d. of 3–6 individual experiments.

strongly coloured too and unfortunately absorb in the same spectral region, making it complicated to determine the kinetics (results not shown). The particular advantages of the EPR assay are that only radicals are observed, and they can be detected at very low concentrations (Rossi et al 1996).

Antioxidant activity of esters

The results obtained with different assays consistently confirm that *ortho*-dihydroxy-4-methylcoumarins are very good radical scavengers; there did not appear to be any differences between 7,8- and 6,7-substituted coumarins, at least in these in-vitro experiments (Table 2). Sharma et al (2005) also reported that the *ortho* position is more favourable than the *meta* position for dihydroxy-4-methylcoumarins, but in their antioxidant assay the 7,8-arrangement was better than the 6,7-substitution. 7,8-Dihydroxy-4-methylcoumarin (C20, 4-methyldaphnetin), which is a natural compound, was previously found to be an excellent antioxidant and particularly efficient against lipid peroxidation (Raj et al 1998a; Liu et al 1999), although this effect seems to be connected with its metal chelation properties (Raj et al 1998c). This compound has also been shown to protect against DNA damage induced by H_2O_2 (Liu & Zheng 2002). It is possible that all these effects are connected to the capacity of C20 to selectively inhibit the pro-inflammatory 5-lipoxygenase, as reported for rat leucocytes by Hoult & Paya (1996). Interestingly, another *ortho*-dihydroxycoumarin, C9 (4-methylesculetin), inhibited 5-lipoxygenase with the same selectivity, whereas the *meta*-dihydroxycoumarin C2 showed a higher inhibitory effect on the leucocyte cyclooxygenase (Hoult & Paya 1996).

A more surprising result was the strong scavenger activity exerted by the *ortho*-diacetoxy-4-methylcoumarins, which were less efficient than the corresponding dihydroxy parent structures, but much better antioxidants than the *meta*-dihydroxy-4-methylcoumarins (Table 2). This behaviour has been noticed before and attributed to the formation of deacetylated compounds through the activity of esterases in biological systems, or to the interaction with lipid ketene structures (Raj et al 1998a). However, in the EPR experiments presented here the samples contained only the diacetoxy compound, the target radical and ethanol, leaving little doubt that the reaction proceeds via one-electron reduction of the radical, excluding even the possibility of a hydrogen-atom-transfer mechanism.

Although the diacetyl esters of dihydroxy-4-methylcoumarins are less active as radical scavengers, they display a wide range of biological effects that seem to be independent of the antioxidant activity. Diacetoxy-4-methylcoumarins inhibit binding of aflatoxin B_1 to DNA in-vitro and thus have potential anti-mutagenic properties; the diesters were found to have much higher activity than the corresponding dihydroxy or dimethoxy compounds (Raj et al 1996, 1998a, c, 2001). The diacetoxy-4-methylcoumarins are also involved in protein acetylation catalysed by transacetylases (Kohli et al 2002; Singh et al 2002; Kumar et al 2005b; Raj et al 2005). The specificity for the transacetylase substrates with respect to the number and position of acetoxy groups on the coumarin ring structure has been examined, and again the most active compounds are those with acetoxy groups in the *ortho* position, particularly 7,8-diacetoxy-4-methylcoumarin (C5). The dihydroxy derivatives are not substrates for these enzymes but are actually the products; at least in this case there is a clear separation of enzymatic and antioxidant effects. In other cases it is not easy to evaluate the antioxidant component of a biological response; both C5 and C20 show good inhibitory activity against ICAM-1 expression (Kumar et al 2005a), and C5 has demonstrated activity as an anti-invasive agent against MCF-7/6 solid tumour cancer cell lines (Parmar et al 2003). All this diverse activity may involve both antioxidant and non-antioxidant mechanisms. Another interesting result is the importance of the substitution at the C-3 position (i.e., on the 2-oxopyran ring), as seen from the data reported in Table 2.

The introduction of the ethoxycarbonylethyl moiety in this position enhanced the radical scavenging capacity of both *meta*- and *ortho*-dihydroxy coumarins, whereas it did not have any effect on the diacetoxy compounds. It is not obvious why a medium-length ester in this position should affect the reductive potential; direct electron-donating or -withdrawing contributions are not likely, but the ester side chain may be sufficiently long to interact with the oxopyran oxygens above the plane of the coumarin ring. This phenomenon could be exploited for the design of improved coumarin antioxidants.

Thionocoumarins may represent a different way of modifying coumarin properties to increase their pharmacological potentials (Kumar et al 2005a). The two 4-methylthionocoumarins tested here did not show much improved radical scavenger activity; the 7,8-dihydroxythionocoumarin (C22) was approximately 10% more active than the corresponding coumarin C20, while the activity of the 7,8-diacetoxythionocoumarin (C21) was not significantly different from its C5 analogue (data not shown). This is in agreement with the finding that thionocoumarins were not more efficient than coumarins in inhibiting microsomal lipid peroxidation (Kumar et al 2005a).

Intracellular antioxidant activity of 4-methylcoumarins

Further experiments were made to verify whether the antioxidant effects of 4-methylcoumarins could be observed directly in cells. The method used was a standard assay based on a fluorescent probe (Pallottini et al 2005). After loading the precursor $DCFH_2$, the cells are exposed to an oxidative stress resulting in the formation of H_2O_2; intracellular peroxidases then use this H_2O_2 to oxidize $DCFH_2$ to the fluorescent DCF. Antioxidants that intercept superoxide or other peroxide-forming radicals prevent the increase in DCF fluorescence.

The *o*-dihydroxycoumarin C9 efficiently decreased ROS production in L6 myoblasts even at very low concentrations, demonstrating that this compound enters the cells and maintains its high scavenger activity in the intracellular environment (Figure 4). In contrast, the dimethoxycoumarin C15 did not affect ROS production, as predicted. None of the coumarins tested gave rise to any detectable cytotoxic effects within the time of the experiments (for C9, which gave the largest decrease in ROS levels, the percentage of living cells at the end of the experiments was 105 ± 19, compared with $100 \pm 13\%$ living cells in control samples). This, of course, does not exclude any potential inhibitory effects of C9 on lipoxygenases or cyclooxygenase in L6 myoblasts, in line with findings reported for leucocytes (Hoult & Paya 1996). Interestingly, the diacetoxycoumarin C5 gave the same protection as C9 against the oxidative stress, although C5 is much less active as a radical scavenger (Table 2). The explanation probably is that inside the cells this compound is rapidly de-esterified through the activity of esterases, thus giving rise to the formation of the excellent scavenger C20. Little is known about the uptake of coumarins in cells, so the esterases involved might actually be the same enzymes responsible for the transacetylation activity mentioned above.

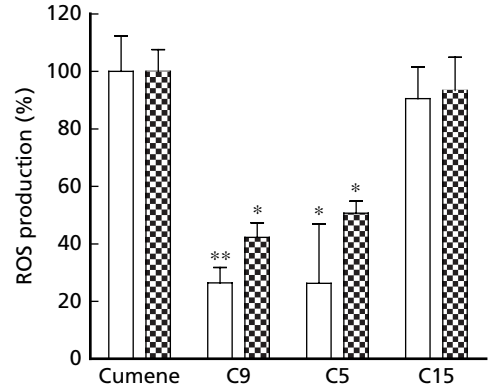

Figure 4 Effect of three different 4-methylcoumarins on the levels of intracellular reactive oxygen species formed in L6 myoblasts after exposure to cumene hydroperoxide. The production of ROS was measured through DCF fluorescence; the coumarin concentrations were 10 μM (left columns) or 1 μM (right columns), the columns marked 'Cumene' show two different control experiments made to define 100% ROS production. Statistical significance of the differences was tested using the Friedman test combined with a Dunn's post-hoc test ($n = 5$). For C9 and C5, the decrease in ROS production was statistically significant at both concentrations; $^*P < 0.05$ or $^{**}P < 0.01$ with respect to the controls.

Conclusions

The results presented in this study outline the structural requirements necessary to design good radical scavengers, and demonstrate that when 4-methylcoumarins are taken up by cells, they maintain their antioxidant capacity and efficiently eliminate intracellular reactive oxygen species. The effects are observed even at coumarin levels in the range of 1–10 μM, corresponding to the concentrations that would be relevant for therapeutic purposes. The results also demonstrate that it is possible to improve the radical scavenging activity of natural and synthetic 4-methylcoumarins by introducing new types of substitutions in the ring structure. Unexpectedly, the addition of an ethoxycarbonylethyl substituent at the C-3 position increased the antioxidant efficiency of already excellent *o*-dihydroxy-4-methylcoumarins; this effect cannot be easily explained by simple electron distribution considerations.

As expected the *o*-dihydroxysubstituted coumarins were excellent radical scavengers and better than the *m*-dihydroxysubstituted or monohydroxysubstituted analogues, but surprisingly the corresponding *o*-diacetoxy derivatives also turned out to be good scavengers, even in the absence of an esterase. Considering that 4-methylcoumarins, in contrast to other coumarins, are not metabolized to toxic epoxide intermediates, these results indicate new promising strategies for the design of non-toxic antioxidant coumarin-based drugs. Our findings prove that 4-methylcoumarin radical scavengers act like biological antioxidants and protect cells against oxidative stress. However, the data in Figure 4 also illustrate that the design of functional antioxidants has to consider factors other than the simple capability to eliminate free radicals in-vitro. For the synthesis of new coumarin derivatives, it will be useful to take into account the information obtained from the

numerous studies on flavonoid molecules (Firuzi et al 2005). Also in flavonoids the substituents influence the antioxidant activity — the greater the number of hydroxyl substitutions, the greater the scavenging activity, with the C-3 hydroxy substitution seeming to be particularly important (Firuzi et al 2004). However, the many contrasting findings concerning the biological functions of natural flavonoid compounds again point to the problem of separating the antioxidant activity of such molecules from all their other possible biological effects.

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