4-Methylcoumarin Derivatives Inhibit Human Neutrophil Oxidative Metabolism and Elastase Activity

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ABSTRACT Increased neutrophil activation significantly contributes to the tissue damage in inflammatory illnesses; this phenomenon has motivated the search for new compounds to modulate their effector functions. Coumarins are natural products that are widely consumed in the human diet. We have evaluated the antioxidant and immunomodulator potential of five 4-methylcoumarin derivatives. We found that the 4-methylcoumarin derivatives inhibited the generation of reactive oxygen species by human neutrophils triggered by serum-opsonized zymosan or phorbol-12-myristate-13-acetate; this inhibition occurred in a concentration-dependent manner, as revealed by lucigenin- and luminol-enhanced chemiluminescence assays. Cytotoxicity did not mediate this inhibitory effect. The 7,8-dihydroxy-4-methylcoumarin suppressed the neutrophil oxidative metabolism more effectively than the 6,7- and 5,7-dihydroxy-4-methylcoumarins, but the 5,7- and 7,8-diacetoxy-4-methylcoumarins were less effective than their hydroxylated counterparts. An analysis of the biochemical pathways suggested that the 6,7- and 7,8-dihydroxy-4-methylcoumarins inhibit the protein kinase C-mediated signaling pathway, but 5,7-dihydroxy-4-methylcoumarin, as well as 5,7- and 7,8-diacetoxy-4-methylcoumarins do not significantly interfere in this pathway of the activation of the human neutrophil oxidative metabolism. The 4-methylcoumarin derivatives bearing the catechol group suppressed the elastase and myeloperoxidase activity and reduced the 1,1-diphenyl-2-picrylhydrazyl free radical the most strongly. Interestingly, the 5,7-dihydroxy-4-methylcoumarin scavenged hypochlorous acid more effectively than the *o*-dihydroxy-substituted 4-methylcoumarin derivatives, and the diacetoxy-4-methylcoumarin derivatives scavenged hypochlorous acid as effectively as the 7,8-dihydroxy-4-methylcoumarin. The significant influence of small structural modifications in the inhibitory potential of 4-methylcoumarin derivatives on the effector functions of neutrophil makes them interesting candidates to develop new drugs for the treatment of inflammatory diseases mediated by increased neutrophil activation.

KEY WORDS: • antioxidant • chemiluminescence • elastase • hypochlorous acid • immunomodulation • 4-methylcoumarin • myeloperoxidase • neutrophil • protein kinase C

INTRODUCTION

NEUTROPHILS CONSTITUTE 40–60% of the white blood cell population in healthy humans and represent the first line of the body's defense against invading pathogens, virally infected cells, and tumor cells.¹ Recognition of microbial components by neutrophils triggers phagocytosis, NADPH oxidase- and myeloperoxidase (MPO)-mediated reactive oxygen species (ROS) generation, and release of granule proteins, such as elastase, cathepsins, and matrix metalloproteinases.^{1,2} These ROS and enzymes act as microbicidal agents, degrade the extracellular matrix, contrib-

ute to cellular migration at the inflammatory site, and regulate the role of leukocytes and lymphocytes in innate and adaptive immune responses.^{1–3}

However, increased neutrophil recruitment and activation are involved in the pathogenesis of a number of autoimmune and inflammatory human diseases, such as systemic lupus erythematosus, rheumatoid arthritis, Graves' disease, Crohn's disease, Behçet's disease, and chronic obstructive pulmonary disease.^{1,2,4,5} Our research group has investigated the antioxidant and immunomodulatory activities of plant extracts^{6–8} and isolated secondary metabolites like coumarins,^{9–11} flavonoids,^{7,12–14} and sesquiterpene lactones.¹⁵ We aim to discover prototypes of compounds that can modulate the effector functions of neutrophils and supplement the endogenous antioxidant defense system.

Coumarins comprise a very large class of compounds that occur in higher plants; they exist in high concentrations in the Rutaceae and Umbelliferae.¹⁶ The human exposure to

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coumarins from dietary sources is quite significant, since these compounds occur in many grasses and papilionaceous plants (Papilionaceae) like tonka bean, woodruff, dates, and certain types of cinnamon. They also occur in green tea, chicory, bilberry, cloudberry, and the essential oils of cinnamon bark, cassia leaf, and lavender. Some coumarins find application in the manufacture of foods, beverages, caramel confectionery, and chewing gum.^{16–18}

Natural and synthetic coumarins and coumarin-related compounds exhibit a variety of biological effects, such as antioxidant, antimicrobial, antitumoral, anticoagulant, antiviral, and anti-inflammatory action.^{16,19} In contrast to many other coumarins, the 4-methylcoumarins are not metabolized to toxic epoxide intermediates.^{17,20} The 4-methylumbelliferone, 4-methyldaphnetin, and 4-methylesculetin are naturally occurring derivatives that scavenge free radicals and inhibit the activity of leukocyte 5-lipoxygenase and lipid peroxidation,^{19–24} revealing the potential anti-inflammatory action of the 4-methylcoumarin derivatives.

Considering the important contribution of neutrophil-derived ROS and enzymes to the inflammatory process, we have evaluated the inhibitory effect of five 4-methylcoumarins on the human neutrophil oxidative metabolism triggered by different signaling pathways, as well as their inhibitory activity toward neutrophil enzymes and their free radical scavenging activity.

MATERIALS AND METHODS

Chemicals

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), lucigenin (*N,N'*-dimethyl-9,9'-biacridinium dinitrate), 2,6-di-*tert*-butyl-4-methylphenol (BHT), cytochalasin B (CB), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *N,N*-dimethylformamide (DMF), phorbol-12-myristate-13-acetate (PMA), quercetin, 3,3',5,5'-tetramethylbenzidine (TMB), gallic acid, taurine, triton X-100, trypan blue, and zymosan A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), ethanol, hydrogen peroxide, *n*-formyl-methionyl-leucyl-phenylalanine (*n*-fMLP), *N*-succinyl-Ala-Ala-Val-*p*-nitroanilide (SAAVNA), methoxy-succinyl-Ala-Ala-Pro-Val-chloromethylketone, human leukocyte MPO (EC 1.7.1.11), and 4-aminobenzoic acid hydrazide were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). Gelatin (microbiological grade) was acquired from Difco Laboratories (Detroit, MN, USA) and lactate dehydrogenase (LDH) LiquiformTM was provided by Labtest Diagnostica (Lagoa Santa, MG, Brazil). All the other chemicals and solvents used in this work were of analytical grade and purchased from commercial sources.

Coumarins

Coumarin (**1**) was purchased from Sigma-Aldrich and the other coumarins (**2**–**6**) were synthesized by the method de-

scribed by Lopes *et al.*²⁵ with slight modifications. The purity of all compounds was 97–100%. Their chemical structures are shown in Figure 1.

Human neutrophil isolation

Twenty adult volunteers who met the criteria described by Paula *et al.*⁸ were recruited for this study. The experimental procedures were approved by the local Research Ethics Committee on Human Experimentation (CEP/FCFRP-USP, protocol 96), according to the rules of the 196/96 Resolution of the Brazilian National Health Council and the Helsinki Declaration of 1975 (revised in 2008).

Blood was collected by venous puncture and diluted 1:2 in the Alsever solution (anticoagulant). Neutrophils were isolated using sterile and lypopolysaccharide-free solutions, as previously described.⁸ The cell pellets were suspended in the Hanks balanced saline solution (HBSS) containing 1 mg/mL gelatin (HBSS-gel). The cell preparations contained 90–95% neutrophils with viability higher than 95%, as established by exclusion with Trypan Blue.

Preparation of the stimuli

Zymosan particles were prepared and opsonized with normal human serum as previously described.¹² Serum-opsonized zymosan (SOZ) was suspended in HBSS for use (10 mg/mL). A 10 mM PMA stock solution was prepared in DMSO, stored at –70°C, and diluted 1:1000 in HBSS before use.⁸

Neutrophil chemiluminescence assay

A 90 mM luminol stock solution was prepared in 0.1 M NaOH and diluted 1:100 in HBSS containing 5 mM HEPES before use.²⁶ Lucigenin was dissolved in HBSS-HEPES. Both probes were prepared daily.

	R ¹	R ²	R ³	R ⁴	R ⁵
1:	H	H	H	H	H
2:	CH ₃	H	OH	OH	H
3:	CH ₃	H	H	OH	OH
4:	CH ₃	OH	H	OH	H
5:	CH ₃	H	H	OCOCH ₃	OCOCH ₃
6:	CH ₃	OCOCH ₃	H	OCOCH ₃	H

FIG. 1. Chemical structures of coumarins.

Reaction mixtures containing neutrophils (1×10^6 cells/mL), the chemiluminescent probe (100 μ M luminol or 100 μ M lucigenin), and a coumarin (0.01–100 μ M) or DMSO (0.1% v/v, control), were preincubated for 3 min, at 37°C. The reaction was initiated by adding SOZ (1 mg/mL) or PMA (0.1 μ M) and the luminol- or lucigenin-enhanced chemiluminescence (CL-lum and CL-luc, respectively), was measured for 15 min, at 37°C, in a luminometer (Auto Lumat LB953; EG&G Berthold, Bad Wildbad, Germany). The light emission was recorded in c.p.m. (photon counts per minute). Background CL produced by nonstimulated cells was subtracted from all the samples. The percentage of CL inhibition by each sample was calculated using the formula $[1 - (\text{AUC}_{\text{sample}}/\text{AUC}_{\text{control}})] \times 100$. AUC is the area under the time-course curve, and it is used to determine IC_{50} values (concentration that inhibits 50% of the neutrophil CL) by nonlinear regression.

Cytotoxicity evaluation

The cytotoxic effect of coumarins on neutrophils was evaluated as previously described.²⁶ Briefly, aliquots of neutrophils (1×10^6 cells/mL) were incubated with a coumarin (200 μ M), DMSO (0.1% v/v, vehicle), HBSS (non-treated cells), or Triton X-100 (0.2% v/v, positive control) for 20 min, at 37°C. The cell pellets were immediately suspended in HBSS gel after centrifugation (755 g, 10 min, 4°C) and cellular viability was determined by the Trypan Blue exclusion test, by counting a total of 200 cells for each sample. The activity of LDH released into the supernatant was measured on the basis of absorbance changes at 340 nm for 2 min, at 37°C (Beckman DU-70 spectrophotometer, Fullerton, CA, USA). The LDH Liquiform test kit was used in this assay.

Elastase activity

The elastase-rich supernatant was obtained from human neutrophils treated with CB and *n*-fMLP, as described by Kanashiro *et al.*¹³ Aliquots of the supernatant were mixed with a coumarin (100 μ M) or DMSO (0.1% v/v, control) and incubated for 10 min, at 37°C. The substrate SAAVNA (330 μ M) was added to all samples, and the absorbance of the product *p*-nitroaniline was recorded at 405 nm in a plate reader (EIA Multi-well Reader II, Sigma Diagnostics, St. Louis, MO, USA), after 30 min of incubation at 37°C. Appropriate blanks without the substrate were subtracted from all the samples.

MPO activity

The effect of coumarins on the MPO activity was evaluated using the method of Kitagawa *et al.*,²⁷ with modifications. The enzyme activity in the MPO stock solution was determined as described by Marquez *et al.*²⁸

Reaction mixtures containing PBS (10 mM phosphate buffer pH 7.4, 140 mM NaCl, 10 mM KCl), luminol (20 μ M), MPO (20 mU/mL), and a coumarin (0.01–10 μ M) or DMSO (0.01% v/v, control) were incubated for 2 min, at

37°C. The reaction was initiated with H_2O_2 (50 μ M), and the CL was measured in a luminometer for 20 min, at 37°C. The IC_{50} values were calculated as described in the neutrophil CL assay.

Hypochlorous acid scavenging assay

The HOCl scavenging potential of coumarins was assessed by the method of Dypbukt *et al.*²⁹ with modifications. Domestic NaOCl was neutralized by dilution in PBS immediately before use. Stock solutions of coumarins were prepared in DMF and diluted in PBS for use. The working solution of a coumarin (0.1–30 μ M) or DMF (0.03% v/v, control) was added to HOCl (50 μ M) and allowed to react for 10 min. Taurine (5 mM) was added to the reaction medium and taurine chloramine was detected after 5 min by rapidly mixing the developing reagent (2 mM TMB in 400 mM acetate buffer pH 5.4 containing 10% DMF and 100 μ M KI). The absorbance was recorded at 650 nm in a plate reader after 5 min of incubation. All the reaction steps were conducted in the dark at 25°C.

DPPH scavenging activity

The DPPH free radical scavenging potential of coumarins was examined by the method of Blois³⁰ with modifications.³¹ The DPPH stock solution was prepared in ethanol and diluted 6:4 in a 40 mM acetate buffer pH 5.5 for use.

The DPPH working solution (100 μ M) was incubated with a coumarin (0.1–100 μ M) or DMSO (0.1% v/v, control) for 5 min, at 25°C, and the absorbance was recorded at 517 nm. The difference between absorbance readings before and after sample addition was considered to calculate the percentage of DPPH reduction by each sample.

Data analysis

Experimental data were processed and analyzed with the aid of the GraphPad Prism Software (version 3.00 for Windows; GraphPad Software, Inc., San Diego, CA, USA). Statistical analysis was performed by Analysis of Variance followed by the Tukey's test. $P < .05$ was considered significant.

RESULTS

Cellular CL inhibition

We used the lucigenin (CL-luc)- and luminol (CL-lum)-enhanced CL assays to evaluate the inhibitory effect of the six coumarins (**1–6**) on the $\text{O}_2^{\bullet-}$ and total ROS production, respectively, by SOZ- or PMA-stimulated human neutrophils.

Coumarins **2–6** inhibited the CL-luc and CL-lum in a concentration-dependent manner (Fig. 2). Coumarins **1** and **6** diminished < 50% CL at the highest concentration tested (100 μ M) (Fig. 2). Table 1 reports the IC_{50} values of coumarins **2–5**. Coumarin **2** suppressed the PMA- and SOZ-stimulated neutrophil CL-lum and CL-luc the most strongly; it was two times more effective than the *o*-dihydroxylated coumarin (**3**)

FIG. 2. Concentration-dependent inhibitory effect of 4-methylcoumarins on the human neutrophil oxidative metabolism. The cells (1×10^6 cells/mL) were stimulated with serum-opsonized zymosan (SOZ, 1 mg/mL; **A, B**) or phorbol-12-myristate-13-acetate (PMA, 0.1 μM; **C, D**), and the luminol (CL-lum; **A, C**) or lucigenin (CL-luc; **B, D**)-enhanced chemiluminescence was measured for 15 min at 37°C. Data are expressed as the mean $\pm$ SD of at least four independent experiments assayed in duplicate.

and at least five times more effective than the *m*-dihydroxylated coumarin (**5**). Acetylating the free hydroxyls of coumarins **3** and **4** decreased their inhibitory potency by more than fourfold (Fig. 2, Table 1).

Coumarins **2–6** inhibited the SOZ-stimulated neutrophil CL-luc more strongly than they inhibited the PMA-stimulated neutrophil CL-luc. An opposite tendency was observed for CL-lum: most of the coumarins (**4–6**) were more potent at inhibiting the PMA-stimulated neutrophil CL-lum.

Coumarins **3** and **5** suppressed the SOZ-stimulated neutrophil CL-lum and CL-luc to the same degree, but coumarins **2** and **4** inhibited the CL-luc more effectively. Coumarins **2–6** inhibited the PMA-stimulated neutrophil CL-lum more strongly than they inhibited the PMA-stimulated neutrophil CL-luc.

Cytotoxicity

Compared with the control, the six coumarins did not induce significant LDH release or decrease neutrophil viability under the assessed conditions (Table 1). Thus, cyto-

toxicity does not underlie the inhibition of neutrophil ROS generation by the tested coumarins.

Inhibition of elastase and MPO activity

The *o*-dihydroxylated coumarins **2** and **3** inhibited the elastase catalytic activity (Table 2). Coumarin **2** inhibited the MPO activity similarly to quercetin and twice more effectively than coumarins **3** and **4**. Acetylating the free hydroxyls decreased their inhibitory potency by 10- to 20-fold (Table 2).

HOCl and DPPH radical scavenging ability

The *o*-dihydroxylated coumarins **2** and **3** scavenged the DPPH radical five times more effectively than the reference antioxidant BHT and the *m*-dihydroxylated coumarin **4**, but twice less effectively than quercetin (Table 3).

Coumarins **2** and **4** scavenged HOCl the most strongly; they were as efficient as quercetin, but twice more efficient than compounds **3**, **5**, and **6** to scavenge HOCl. BHT and

TABLE 1. EFFECT OF 4-METHYLCOUMARINS ON HUMAN NEUTROPHIL REACTIVE OXYGEN SPECIES GENERATION AND CELLULAR VIABILITY

Compound	ROS generation—IC ₅₀ (μM)*				Cytotoxicity†	
	SOZ		PMA		Viable cells (%)‡	LDH release (%)§
	CL-lum	CL-luc	CL-lum	CL-luc		
HBSS	—	—	—	—	97.5 ± 2.8	4.9 ± 2.1
DMSO	—	—	—	—	97.5 ± 1.7	4.5 ± 1.8
1	> 100	> 100	> 100	> 100	96.3 ± 2.3	4.5 ± 1.5
2	8.98 ± 0.61 ^{aA}	5.65 ± 0.91 ^{aB}	8.63 ± 1.06 ^{aA}	12.76 ± 1.15 ^{aC}	98.5 ± 0.6	3.7 ± 1.3
3	4.01 ± 0.52 ^{bA}	3.66 ± 0.53 ^{bA}	3.74 ± 0.53 ^{bA}	7.01 ± 1.16 ^{bB}	97.8 ± 1.0	5.3 ± 1.0
4	34.17 ± 2.36 ^{cA}	27.60 ± 3.02 ^{cB}	18.69 ± 1.05 ^{cC}	84.56 ± 4.28 ^{cD}	97.6 ± 0.9	4.8 ± 1.5
5	18.83 ± 1.62 ^{dA}	16.47 ± 2.51 ^{dAB}	13.08 ± 1.64 ^{dB}	> 100	98.1 ± 0.8	4.1 ± 1.2
6	> 100	> 100	> 100	> 100	98.0 ± 1.6	4.5 ± 2.3

*Neutrophils (1 × 10⁶ cells/mL) were stimulated with SOZ (1 mg/mL) or PMA (0.1 μM), and the luminol- or lucigenin-enhanced chemiluminescence (CL-lum and CL-luc, respectively) was measured for 15 min at 37°C. IC₅₀ values are expressed as mean ± SD of at least four independent experiments, assayed in duplicate.

†Cytotoxicity data represent the mean ± SD of three independent experiments assayed in duplicate. Samples **1–4** and **6** were tested at 200 μM, and sample **5** was tested at 100 μM (due to low solubility in the reaction medium).

‡Determined by the Trypan Blue exclusion test.

§LDH release by the samples was compared with neutrophils completely lysed by Triton X-100.

Values in a column^(abcd) or in a row^(ABCD) not sharing the same letter are significantly different from each other (ANOVA and Tukey's *post hoc* test, *P* < .05).

SOZ, serum-opsonized zymosan; PMA, phorbol-12-myristate-13-acetate; SD, standard deviation; IC₅₀, concentration inhibiting CL-lum or CL-luc by 50%; HBSS, Hank's balanced salt solution (untreated cells); DMSO, dimethyl sulfoxide (0.1% v/v; vehicle control); ANOVA, analysis of variance.

coumarin **1** did not scavenge HOCl significantly at 30 μM, the highest concentration tested (Table 3).

DISCUSSION

The results reported here are part of a continuous study of the pharmacological activities of plant extracts and isolated compounds on the effector functions of neutrophils.^{6–15} In the present study, we assessed whether six coumarin derivatives inhibit the human neutrophil oxidative metabolism

and elastase activity, and investigated some of the underlying mechanisms of action of these compounds.

To gain an initial insight into the action of the tested coumarins in the neutrophil oxidative metabolism, we used two stimuli—PMA and SOZ. The former activates protein kinase C (PKC) directly;³² the last is a model phagocytic stimulus that binds to the complement, IgG, and mannose membrane receptors of neutrophils.³³ SOZ triggers complex intracellular signaling pathways involving phospholipases and kinases, due to engagement of multiple receptors, but PKC translocation and activation is an important early event of the NADPH oxidase assembly triggered by SOZ.³³ The experimental strategy also involves the use of the chemiluminescent probes lucigenin (CL-luc) and luminol (CL-lum) to study how the coumarins affect different steps of the neutrophil ROS generation process. Lucigenin specifically detects O₂^{•−}, the first ROS produced via the NADPH oxidase complex; luminol measures the overall ROS generation, with higher sensitivity for the ROS produced via the MPO–H₂O₂–halide system in subsequent steps.³⁴

We found that the hydroxylated and acetylated 4-methylcoumarins (**2–6**) inhibit the SOZ- or PMA-stimulated neutrophil ROS generation in a concentration-dependent way. The presence of an oxygenated substituent is essential to suppress this neutrophil function by 4-methylcoumarins, as reported for simple coumarins and 3-phenylcoumarins.^{9,11} In addition, toxicity of the 4-methylcoumarins on these cells does not mediate inhibition of the neutrophil oxidative metabolism, at least under the assessed conditions.

Coumarins **2** and **3** inhibit the two steps of the neutrophil ROS generation the most effectively; they also give similar IC₅₀ values for PMA- and SOZ-stimulated neutrophils, CL-luc and CL-lum. These results suggest that coumarins **2** and **3** suppress an early event of the neutrophil oxidative

TABLE 2. INHIBITION OF HUMAN NEUTROPHIL ELASTASE AND MYELOPEROXIDASE ACTIVITY BY 4-METHYLCOUMARINS

Compound	Inhibition of elastase activity (%)*	Inhibition of MPO activity	
		at 20 μM (%)	IC ₅₀ (μM)
1	−0.87 ± 2.33 ^a	5.32 ± 0.56 ^a	> 20
2	17.15 ± 0.63 ^b	99.82 ± 0.06 ^b	0.67 ± 0.12 ^a
3	21.13 ± 2.16 ^b	99.93 ± 0.04 ^b	1.10 ± 0.20 ^b
4	5.85 ± 0.64 ^c	99.86 ± 0.08 ^b	1.06 ± 0.16 ^b
5	4.50 ± 0.71 ^c	94.03 ± 3.44 ^b	9.27 ± 0.47 ^c
6	7.37 ± 3.63 ^c	49.24 ± 3.56 ^c	> 20
Quercetin	n.t.	99.75 ± 0.10 ^b	0.68 ± 0.09 ^a
AAPV†	87.6 ± 3.4 ^d	n.t.	n.t.
ABAH‡	n.t.	99.68 ± 0.06 ^b	0.04 ± 0.01 ^d

Data expressed as mean ± SD of three independent experiments assayed in duplicate.

*Coumarins tested at 100 μM.

†AAPV (a specific inhibitor of elastase activity) tested at 1 μM.

‡ABAH is a specific inhibitor of MPO activity.

^{abcd}Values in a column not sharing the same letter are significantly different from each other (*P* < .05; ANOVA and Tukey's *post hoc* test).

MPO, myeloperoxidase; AAPV, methoxy-succinyl-Ala-Ala-Pro-Val-chloromethylketone; ABAH, 4-aminobenzoic acid hydrazide; n.t., not tested.

TABLE 3. ANTIOXIDANT EFFECT OF 4-METHYLCOUMARINS IN CELL-FREE SYSTEMS

Compound	DPPH reduction		HOCl scavenging	
	at 100 μ M (%)	IC ₅₀ (μ M)	at 30 μ M (%)	IC ₅₀ (μ M)
1	-3.81 ± 1.07^a	> 100	7.38 ± 2.21^a	> 30
2	80.78 ± 1.76^b	23.61 ± 2.62^a	98.26 ± 2.96^b	4.08 ± 0.79^a
3	80.52 ± 1.82^b	23.07 ± 2.22^a	99.00 ± 5.30^b	8.22 ± 0.72^{bc}
4	18.10 ± 2.23^c	> 100	98.58 ± 1.13^b	2.85 ± 0.13^a
5	-1.84 ± 1.56^a	> 100	87.30 ± 2.57^c	9.47 ± 1.43^b
6	-2.76 ± 1.79^a	> 100	96.56 ± 2.50^b	7.27 ± 0.52^c
BHT	42.64 ± 2.24^d	125.70 ± 5.94^b	16.36 ± 7.37^d	> 30
quercetin	81.83 ± 2.19^b	9.28 ± 0.27^c	96.93 ± 0.49^b	3.03 ± 0.25^a

Data expressed as mean $\pm$ SD of three independent experiments assayed in duplicate.

^{abcd}Values in a column not sharing the same letter are significantly different from each other ($P < .001$; ANOVA and Tukey's *post hoc* test).

DPPH, 1,1-diphenyl-2-picrylhydrazyl; BHT, 2,6-di-*tert*-butyl-4-methylphenol.

metabolism activation and that the signaling pathway is common to both stimuli. A possible target is the PKC translocation and activation, which is essential for NADPH oxidase assembly and $O_2^{\bullet-}$ production. Decreased $O_2^{\bullet-}$ generation reduces the availability of H_2O_2 , which is a substrate for MPO-catalyzed reactions, consequently diminishing CL-lum. The $O_2^{\bullet-}$ scavenger effect of coumarins **2** and **3**^{19,23} may also contribute to CL-luc and CL-lum inhibition.

Coumarins **4** and **5** do not significantly inhibit the PMA-stimulated neutrophil CL-luc, suggesting that these compounds do not suppress the PKC activity. On the other hand, these coumarins inhibit the PMA-stimulated neutrophil CL-lum and the SOZ-stimulated neutrophil CL-luc and CL-lum, indicating that they modulate another signaling pathway. The phosphatidylinositol-3-kinase (PI3-K) pathway is a possible target, since it mediates the granule fusion events that are essential for the MPO activity and CL-lum production by the PMA- and SOZ-stimulated neutrophils.^{32,33,35}

Modulating the activity of protein kinases can be effective for the treatment of many diseases. In recent years, there are many protein kinase inhibitors being tested in clinical trials for cancer, cardiovascular diseases, prevention of transplant rejection, and autoimmune diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel diseases.^{36–38} However, these compounds must be highly specific and selective toward the target protein kinase to minimize adverse effects, because there are a large number of kinases in the human genome and many of them play important roles in pathways that regulate the normal cellular processes.^{36,37} The effect of the 4-methylcoumarin derivatives on the subclasses of protein kinases remains to be investigated.

The presence of free dihydroxyl groups determines the ability of 4-methylcoumarins to inhibit the neutrophil oxidative metabolism and the MPO and elastase activity, as well as scavenge free radicals. For instance, the *o*-dihydroxy-substituted 4-methylcoumarins suppressed the neutrophil oxidative metabolism more than the *m*-dihydroxy-substituted 4-methylcoumarins. The 7,8-dihydroxyl group contributed two times more than the 6,7-dihydroxyl group to

the inhibitory effect. Hoult *et al.*¹⁹ reported that the $O_2^{\bullet-}$ scavenging effect of the 7,8-, 6,7-, and 5,7-dihydroxy-4-methylcoumarins follows the same ranking order. The *o*-dihydroxyl group is important for other pharmacological effects of coumarins. Daphnetin (7,8-dihydroxycoumarin) and esculetin (6,7-dihydroxycoumarin), isolated from medicinal plants used to treat allergic and inflammatory diseases, and their 4-methyl analogues, inhibit the rat leukocyte lipoxygenase activity.^{19,24} Esculetin suppresses the tyrosine kinase and PI3-K activity of MCF-7 cells.¹⁹ The 4-methyl-5,7-dihydroxycoumarin strongly inhibits the leukocyte cyclooxygenase activity.¹⁹ Moreover, flavonoids bearing a catechol group inhibit the human neutrophil degranulation.¹³

Esterification of hydroxyl groups can improve the pharmacological properties of phenolic compounds in cell-mediated oxidative and inflammatory processes. This structural modification of flavonoids and coumarins enhances inhibition of prostaglandin synthesis and nitric oxide production by macrophages and prevents intercellular adhesion molecule-1 expression by endothelial cells.^{39,40} Acetylation of quercetin³⁹ and fluorescent probes⁴¹ increases their lipophilicity and intracellular bioavailability. The ester form of these compounds readily crosses the plasma membrane, intracellular esterases cleave the ester bond, and the hydroxylated products accumulate in the cytoplasm. Interestingly, acetylation of free hydroxyl groups of coumarin derivatives can have a positive or negative impact on their ability to inhibit the neutrophil ROS generation, depending on the number and position of the substituents. The diacetylated 4-methylcoumarins **5** and **6** were less effective at inhibiting the PMA- and SOZ-stimulated neutrophil ROS generation than their hydroxylated analogues (**3** and **4**, respectively). The tri- or tetra-acetylated 3-phenylcoumarin derivatives suppressed this neutrophil function more effectively than their hydroxylated counterparts.¹¹

Regarding the structure activity analysis of MPO activity inhibition by the 4-methylcoumarins, (i) the most active coumarin (**2**) bears the 6,7-dihydroxyl group; (ii) the 7,8- and 5,7-dihydroxyl groups (**3**, **4**) equally contribute to the inhibitory effect; (iii) acetylation of free hydroxyls (**5**, **6**)

decreases the inhibitory effect. The free *o*-dihydroxyl group is also the structural requirement for the 4-methylcoumarins to inhibit the elastase activity. These results agree with previous works that reported that the catechol group of flavonols and 3-phenylcoumarins are important to suppress the elastase and horseradish peroxidase activity.^{13,14} Considering that MPO and HOCl are the most important mediators of luminol oxidation in activated neutrophils,³⁴ both inhibition of MPO activity and HOCl scavenging by the 4-methylcoumarin derivatives contribute to decrease the CL-lum produced by these cells and by the cell-free MPO–H₂O₂–halide-luminol system.

The 6,7- and 7,8-dihydroxyl groups (**2**, **3**) equally contribute to reducing the DPPH free radical. They are more efficient free radical scavengers than the *m*-dihydroxy-substituted analogue **5** and the reference antioxidant BHT, agreeing with previous reports.^{10,20,21} On the other hand, 6,7- and 5,7-dihydroxysubstituted 4-methylcoumarins (**2**, **4**) are better HOCl scavengers than the 7,8-dihydroxysubstituted coumarin **3**. The natural compound 7,8-dihydroxy-4-methylcoumarin is a good antioxidant against lipid peroxidation, due to its metal chelation properties.^{21,22} The *o*-dihydroxy system is able to form a resonance-stable radical and yield less reactive quinone or semiquinone products, which explains the excellent radical scavenging ability of compounds bearing the catechol group.¹⁶

Interestingly, the acetylated 4-methylcoumarins do not display remarkable DPPH reducing ability, but they are as good as the 7,8-dihydroxy-4-methylcoumarin to scavenge HOCl. Our results agree with reports of strong free radical scavenger activity of *o*-diacetoxy-4-methylcoumarins, which can be similar to the corresponding dihydroxy parent structures.^{20–22} The concentration of phenolic compounds in the samples of the acetylated 4-methylcoumarins is not significant, so the antioxidant effect of these compounds does not stem from contamination by hydroxylated compounds. However, the mechanism underlying the antioxidant effect of acetylated compounds remains unexplained.

Dietary exposure to coumarins is significant. It is estimated that the average Western diet contains ~1 g/day of mixed benzopyrones, which includes coumarins and flavonoids.¹⁸ Coumarins exist at high levels in some essential oils, particularly the cinnamon bark (7000 ppm) and cassia leaf (up to 87,300 ppm) oil.¹⁷ The human plasma concentration of total polyphenol metabolites ranges from 0.1 to 4 μM with an intake of 50 mg of aglycone equivalents⁴² and can reach 10 μM after a polyphenol-rich meal.⁴³ Some coumarinic drugs are rapidly absorbed from the gastrointestinal tract and distributed throughout the body after oral administration; the absorption rate of coumarin can reach up to 83%.^{17,18} Interestingly, most of the biological effects of the 4-methylcoumarin derivatives tested herein occurred in a range of concentrations considered relevant for therapeutic purposes (1–10 μM).²⁰ Therefore, it is possible that the 4-methylcoumarin derivatives achieve significant pharmacological effects *in vivo*, but further studies are needed to establish the best dosage form to attain therapeutic levels of these compounds. Recently, Witaicenis *et al.*⁴⁴ reported that

4-methylesculetin administered orally displayed antioxidant and intestinal anti-inflammatory effect in rats with acute colitis. These authors suggested that absorption of 4-methylesculetin in the small intestine determined its therapeutic effects, and that the presence of the 4-methyl group improved these effects, in comparison to esculetin.

In summary, the results of the present study contribute to understanding how the 4-methylcoumarins inhibit the human neutrophil oxidative metabolism triggered by different signaling pathways. The structure–activity relationships regarding inhibition of the neutrophil ROS generation process and MPO and elastase activity, as well as the HOCl scavenging action, may contribute to developing more selective compounds. Because 4-methylcoumarins are not metabolized to toxic intermediates, they are interesting therapeutic adjuvants in the treatment of inflammatory diseases involving neutrophils. Further investigation is required to elucidate the molecular mechanisms underlying the pharmacological effect of 4-methylcoumarin derivatives and determine the potential clinical usefulness of these compounds in the adjunctive therapy of inflammatory diseases.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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