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3-Phenylcoumarin derivatives selectively modulate different steps of reactive oxygen species production by immune complex-stimulated human neutrophils

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ABSTRACT

Immune complex (IC) deposition in tissues triggers the release of harmful oxidant and lytic compounds by neutrophils. We examined how ten 3-phenylcoumarin derivatives affect the reactive oxygen species (ROS) production by IC-stimulated human neutrophils. Most of the 3-phenylcoumarins inhibited the luminol-enhanced chemiluminescence (CL-lum) more strongly than they inhibited the lucigenin-enhanced chemiluminescence (CL-luc), without clear signs of toxicity. The most effective CL-lum inhibitors, 6,7-dihydroxy-3-[3′,4′-methylenedioxyphenyl]-coumarin (**5**) and 6,7-dihydroxy-3-[3′,4′-dihydroxyphenyl]-coumarin (**19**), also inhibited myeloperoxidase activity more potently and had higher hypochlorous acid scavenging ability, but did not affect the NADPH-oxidase activity. The type, number, and position of the substituent influenced the pharmacological effects of 3-phenylcoumarins; however, the structural requirements for CL-lum and CL-luc inhibition were a little different. Compounds **5** and **19** are promising prototypes of therapeutic molecules to modulate ROS production by neutrophils in IC-mediated inflammatory diseases.

1. Introduction

The organism continuously produces antigen–antibody immune complexes (IC) in response to foreign antigens, infection, and tissue injury. By binding to erythrocytes, IC are transported to the spleen and liver and further cleared from the circulation. However, increased IC formation and defects in the IC clearance mechanism culminate in IC deposition in vessels and tissues, which activates the complement system and chemotaxis of inflammatory cells [1,2]. Fcγ and complement receptors mediate the neutrophil activation at the inflammatory site, triggering phagocytosis, degranulation, and reactive oxygen species generation (ROS) via the NADPH oxidase complex and myeloperoxidase (MPO) [3]. Microbial killing depends on inflammatory cells releasing oxidant and lytic compounds; however, these compounds can damage the surrounding tissues and impair the target organ function [4]. For instance, IC deposition in tissues is strongly associated with rejection of transplanted organs and initial response of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and rheumatic fever [2].

Neutrophils play a fundamental role in host defense and acute inflammatory responses. To regulate autoimmune inflammation, it is important that neutrophils produce adequate amounts of ROS. Moreover, neutrophils have an important part in chronic inflammatory responses [5–7]. In this sense, modulating neutrophil ROS production is a promising therapeutic strategy to manage inflammation and reduce the deleterious effects of the over-activation of immune system cells in many diseases [5,8,9]. Our research team has investigated the pharmacological effects of natural and synthetic compounds like flavonoids [10–12], coumarins [13–15], and sesquiterpene lactones [16]. We have sought to understand structure–activity relationships, unravel the mechanisms of action of these compounds, and discover prototypes of compounds that can act as modulators of the effector functions of neutrophils in IC-mediated inflammatory diseases.

Abbreviations: AUC, area under the curve; CL, chemiluminescence; CL-luc, lucigenin-enhanced chemiluminescence; CL-lum, luminol-enhanced chemiluminescence; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; DPI, diphenyleneiodonium chloride; HBSS, Hank's balanced saline solution; HBSS-gel, Hank's balanced saline solution supplemented with 0.1% gelatin; IC, immune complex; IC_{50}, concentration inhibiting a biological response by 50%; LDH, lactate dehydrogenase; MPO, myeloperoxidase; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; *p*-AB, *p*-aminobenzohydrazide; ROS, reactive oxygen species; TMB, 3,3′,5,5′-tetramethylbenzidine.

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Coumarins constitute an important class of secondary metabolites that occur in plants and microorganisms, and they display a wide range of biological activities [17,18]. The last ten years have seen intense work on the chemical modification of the coumarin skeleton, in order to obtain more potent antioxidant and anti-inflammatory compounds [13,15,19–23]. The addition of a 3-phenyl ring and hydroxyl groups to the coumarin molecule yields hydroxylated 3-phenylcoumarin derivatives, known for their increased antioxidant effect [14,24] and ability to inhibit peroxidases [14] and lipoxygenase [24]. These properties reveal the potential anti-inflammatory activity of these derivatives.

Higher plants produce 3-phenylcoumarins by the same biosynthetic pathway that flavonoids are generated, so these coumarins are considered a subclass of isoflavonoids [25]. 3-Phenylcoumarins exhibit antimicrobial [26,27], antiviral [28], antidepressant [29], anticoagulant [30], and vasorelaxant [31] actions. Furthermore, they inhibit the glyceraldehyde 3-phosphate dehydrogenase of *Trypanosoma cruzi* [32], monoamine oxidase [33] and tyrosinase [34].

Recently, we screened the inhibitory effect of a series of 3-phenylcoumarin derivatives in the oxidative metabolism of rabbit IC-stimulated neutrophils [15]. The qualitative and quantitative structure–activity relationship analysis allowed us to select promising modulators of this neutrophil effector function. To continue investigating the immunomodulating potential of 3-phenylcoumarins, in this study we evaluated the inhibitory effect of ten selected 3-phenylcoumarin derivatives bearing free and acetylated hydroxyl groups on $O_2^{\bullet -}$ generation and total ROS production by IC-stimulated human neutrophils. We also investigated possible mechanisms underlying the pharmacological effect of these compounds, such as their inhibitory activity toward NADPH oxidase and MPO as well as their hypochlorous acid (HOCl) scavenging and cytotoxic potential.

2. Results

2.1. Inhibition of ROS production by neutrophils

First, we used the CL-luc and CL-lum assays to screen the inhibitory effect of ten 3-phenylcoumarin derivatives (at 20 μM) on the $O_2^{\bullet -}$ and total ROS generation by IC-stimulated neutrophils, respectively. Compared with the control, all the tested compounds had a significant inhibitory effect on both CL-lum and CL-luc (Fig. 2a and b). In general, the 3-phenylcoumarin derivatives inhibited the neutrophil CL-lum more strongly than they inhibited the neutrophil CL-luc; only compounds **1**, **7**, and **9** inhibited CL-lum and CL-luc to the same degree.

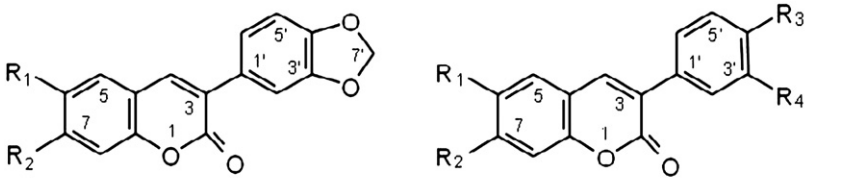
Next, we selected the compounds that inhibited CL-lum (**1**, **5**, **6**, **19**, and **20**) or CL-luc (**1**, **5**, **19**, and **20**) by more than 50% and investigated their mechanism of action. The aforementioned derivatives inhibited the IC-stimulated neutrophil CL-lum (Fig. 2c) and CL-luc (Fig. 2d) in a concentration-dependent manner; their IC_{50} values are reported in Table 2.

Compounds bearing the catechol group (**5** and **19**) inhibited CL-lum the most effectively, but acetylation of their free hydroxyls decreased their inhibitory potency by three- to fourfold (**5** *vs.* **6**; **19** *vs.* **20**) (Table 2). Compounds **5** and **19** had significantly lower IC_{50} values than quercetin. The non-substituted compounds **7** and **8** and the monosubstituted compounds **9** and **10** were the least active — they inhibited nearly 30% of CL-lum. Compounds **1** and **2** inhibited around 70% and 45%, respectively, of CL-lum at the highest concentration tested herein (20 μM) (Fig. 2a).

Compound **5** inhibited CL-luc the most strongly: it was three times more effective than quercetin and five to six times more effective than compounds **1**, **19**, and **20**, which had similar inhibitory effects (Table 2). The hydroxylated 3-phenylcoumarin derivatives had significantly higher inhibitory activity than their acetylated counterparts (**1** *vs.* **2**; **5** *vs.* **6**; **9** *vs.* **10**) (Fig. 2b). Compounds **8** and **10** were the least effective. Compounds **2**, **6**, **7**, and **9** inhibited only 30–40% of CL-luc at 20 μM (Fig. 2b).

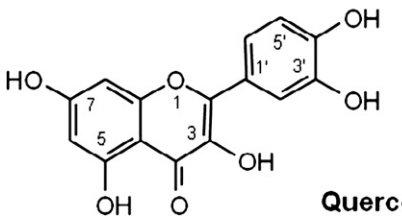
For most of the 3-phenylcoumarin derivatives, the presence of the 3′,4′-methylenedioxyl group enhanced the inhibition of neutrophil CL-luc (**1** *vs.* **9**; **2** *vs.* **10**; **5** *vs.* **19**; **7** *vs.* **8**). In the case of the monosubstituted 3-phenylcoumarin derivatives, this same substituent increased the inhibition of neutrophil CL-lum (**1** *vs.* **9**; **2** *vs.* **10**) (Fig. 2, Table 2).

Together, these results suggest that the type, number, and position of the substituent influence the inhibitory effect of 3-phenylcoumarins on ROS production by IC-stimulated neutrophils. However, the structural



1: $R_1 = OH,\ R_2 = H$
2: $R_1 = OCOCH_3,\ R_2 = H$
5: $R_1 = R_2 = OH$
6: $R_1 = R_2 = OCOCH_3$
7: $R_1 = R_2 = H$

8: $R_1 = R_2 = R_3 = R_4 = H$
9: $R_1 = OH,\ R_2 = R_3 = R_4 = H$
10: $R_1 = OCOCH_3,\ R_2 = R_3 = R_4 = H$
19: $R_1 = R_2 = R_3 = R_4 = OH$
20: $R_1 = R_2 = R_3 = R_4 = OCOCH_3$,



Quercetin

Fig. 1. Chemical structures of 3-phenylcoumarin derivatives and quercetin.