Different effect of two synthetic coumarin-stilbene hybrid compounds on phagocyte activity

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Abstract

OBJECTIVE: Activated phagocytes, generating a variety of powerful inflammatory mediators, such as oxygen and nitrogen species, may participate in oxidative stress-mediated inflammation and organ toxicity. At present, great attention is devoted to the important class of phenolic compounds – coumarins – due to their antiinflammatory/antioxidant activities. We compared two synthetic phenylcoumarins: 7-hydroxy-3-(4´-hydroxyphenyl) coumarin (HHC; 0.01–100 μmol/l) and its hydrogenated analogue: 7-hydroxy-3-(4´-hydroxyphenyl)-3,4-dihydrocoumarin (HHDC; 0.01–100 μmol/l) as their ability to inhibit reactive oxygen species (ROS) generation in human neutrophils and nitric oxide (NO) production by RAW 264.7 macrophages in vitro, with respect to some of their physicochemical characteristics. **METHODS:** ROS production was measured with luminol-enhanced chemiluminescence (CL) in the microplate luminometer Immunotech LM-01T, nitrite formation was determined by the Griess reaction – spectrophotometrically. The radical scavenging assays were employed to assess the antiradical activity values. The relevant physico-chemical parameters of the compounds tested, electronic and hydrophobic, were determined experimentally as well as by suitable computational programmes. **RESULTS:** Both HHC and HHDC were found to decrease significantly ($p<0.01$) CL of whole blood stimulated with phorbol myristate acetate (PMA) from the concentration of 1 μmol/l. While HHC significantly inhibited CL stimulated by A23187 and opsonized zymosan (OpZ), HHDC was ineffective. Unlike HHDC, HHC in the concentrations of 10 and 100 μmol/l significantly ($p<0.01$) reduced NO formation in lipopolysaccharide (LPS) -stimulated murine macrophages RAW 264.7. HHC possessed the higher free radical reducing efficacy in accordance with its more favourable values of electronic parameters in comparison with HHDC. **CONCLUSIONS:** Our results show the different inhibitory effects of HHC and HHDC on phagocytic activity that might be the result of their diverse free radical scavenging properties and lipophilicity features.

ORIGINAL ARTICLE

Abbreviations:

ROS	- reactive oxygen species
NO	- nitric oxide
HHC	- 7-hydroxy-3-(4´-hydroxyphenyl) coumarin
HHDC	- 7-hydroxy-3-(4´-hydroxyphenyl)-3,4-dihydrocoumarin
CL	- chemiluminescence
PMA	- phorbol myristate acetate
OpZ	- opsonized zymosan
LPS	- lipopolysaccharide

INTRODUCTION

The involvement of phagocyte-derived reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the pathophysiology of many inflammatory diseases has been attracting interest in the discovery and synthesis of new compounds with antioxidant and immunomodulatory properties (Kabeya *et al.* 2008). Natural as well as synthetic coumarins have been demonstrated to have antiinflammatory activities via their ability to modulate ROS and RNS production by phagocytes and the expression of various genes involved in inflammatory response, including iNOS, COX-2 and TNF-α (Hoult & Payá 1996; Nakamura *et al.* 2009a;b). Besides antiinflammatory activities, coumarins possess multiple biological effects, such as anticoagulating, anticancer, antimutagenic, antibacterial and antiviral. Many of these beneficial effects may be connected with their antioxidant properties (Beillerot *et al.* 2008; Lin *et al.* 2008). On the other hand, the antioxidant activities are related to the structures of coumarins (Zhang & Wang 2004; Kabeya *et al.* 2007, 2008; Thuong *et al.* 2010).

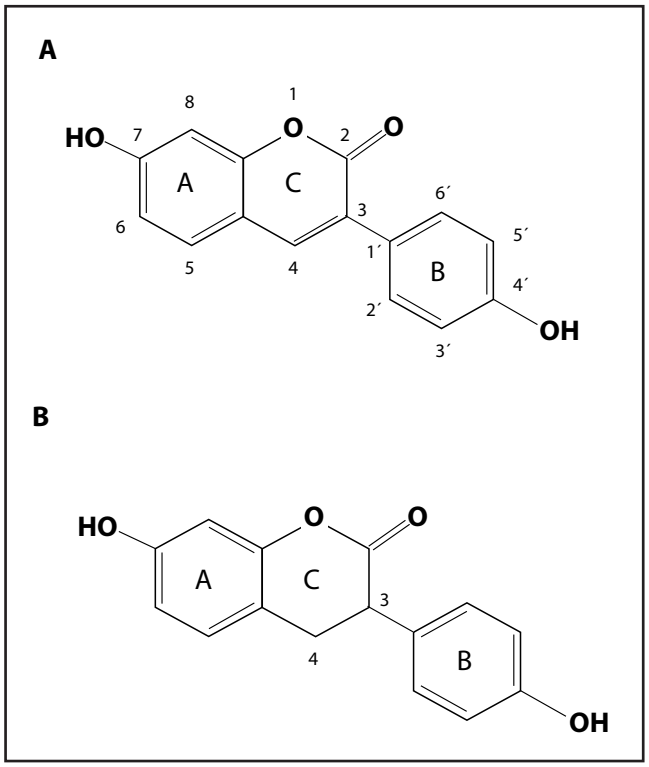

Figure 1 Chemical structure of synthetic phenylcoumarin derivatives related to the natural 7-hydroxycoumarin (umbelliferon). **A**: 7-hydroxy-3-(4´-hydroxyphenyl) coumarin (HHC), **B**: 7-hydroxy-3-(4´-hydroxyphenyl)-3,4-dihydrocoumarin.

We studied the ability of two synthetic coumarin derivatives (Figure 1), HHC (0.01–100 μmol/l) and HHDC (0.01–100 μmol/l) to inhibit: a) reactive oxygen species (ROS) generation in human neutrophils stimulated with phorbol-12-myristate acetate (PMA receptor bypassing stimulus), opsonized zymosan (OpZ receptor-operating stimulus) and calcium ionophore (A 23187 receptor bypassing stimulus), and b) nitric oxide (NO) production by RAW 264.7 macrophages stimulated with LPS. Biological effects of both coumarins we compared with their relevant physicochemical characteristics and free radical scavenging activities in chemical assays.

MATERIALS AND METHODS

Both phenylcoumarins, HHC and its dihydro derivative HHDC, were prepared by synthesis using original approach (Šmidrkal *et al.* 2010), modified for producing stilbene carboxyl intermediates (Harmatha *et al.* 2008), sequentially forming the final lactone ring C (Figure 1). Phorbol-myristate-13-acetate (PMA), luminol (5-amino-2,3-dihydro-1,4 phthalazinedione), A23187, zymosan A from *Sacharomyces cerevisiae*, Griess reagent, 1,1´-diphenyl-2-picrylhydrazyl (DPPH·) radical and ABTS [2,2´-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid)] were from Sigma-Aldrich Chemie (Deisenhofen, Germany), Horse radish peroxidase (HRP) from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS) contained 137 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4 and 1.5 mmol/l KH_2PO_4, pH 7.4. Reception of blood samples from the National Transfusion Service, Bratislava, Slovak Republic is greatly acknowledged. This work was approved by the Local Ethic Committee, Institute of Experimental Pharmacology and Toxicology SAS.

Blood collection and chemiluminescence assay of human whole blood

Fresh blood was taken at the blood bank from healthy volunteers (men, aged 20 to 50 years) by antecubital venepuncture. Effect of HHC (0.01, 0.1, 1, 10, 100 μmol/l) or HHDC (0.01, 0.1, 1, 10, 100 μmol/l) on ROS generation in whole blood (250 × diluted) was measured by using luminol (250 μmol/l) enhanced CL after stimulation with PMA (0.05 μmol/l) or A23187 (1 μmol/l) or opsonized zymosan (OpZ – 0.5 mg/ml). CL was evaluated in a microplate luminometer Immunotech LM-01T (Czech Republic) at 37 °C. Data were based on integral values of CL over 3 600 s (RLU × s; RLU relative light units) (Drábiková *et al.* 2009).

Cell culture

Murine peritoneal macrophage cell line RAW 264.7 was cultivated in Dulbecco's Modified Eagle Medium

and supplemented with 10% of foetal bovine serum. Cells were maintained at 37 °C, 5% CO_2 (Pekarova *et al.* 2009).

Measurement of nitrite concentration by Griess reaction

The presence of nitrite was determined as a stable oxidised product of NO in cell media by the Griess method. Briefly, RAW 264.7 cells (1×10^6 cells/well) were incubated in 12-well plates for 20 h with LPS (0.1 μg/ml) and HHC or HHDC at 37 °C, 5% CO_2. Control cells were incubated with LPS without the substances tested. At the end of the incubation period, culture media were collected from wells and centrifuged at 5,000 × *g* and 4 °C for 5 min. Culture supernatant (150 μl) was mixed with an equal volume of Griess reagent in a 96-well plate and the mixture was incubated at room temperature in the dark for 30 min. The absorbance was measured at 546 nm. Sodium nitrite was used as standard (Ambrozova *et al.* 2010).

Determination of R_M values

The lipophilicity parameters represented by R_M values were measured by reversed-phase thin layer chromatography (RP TLC) technique. The mobile phase consisted of dilute acetic acid (pH 2.5) mixture with acetonitril (20:80, v:v) (Király-Véghely *et al.* 2004). The stationary phase was obtained by impregnation of the layer of Silica gel G F254 plates with 5% solution of liquid paraffin in ether. The R_M values were calculated by the formula: $R_M=\log(1/R_F-1)$ (Račková *et al.* 2006).

DPPH and ABTS·+ free radical scavenging assay

The reactivity of HHC and HHDC (25 μmol/l) toward the stable free 1,1´-diphenyl-2-picrylhydrazyl (DPPH) radical and the reactivity of HHC and HHDC (12.5 μmol/l) toward ABTS·+ [2,2´-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) radical cation] was evaluated as initial rates of absorbance decreases of ethanolic solution at 518 nm and phosphate buffer solution (pH 7.4) at 734 nm, respectively, following addition of the compounds tested (Račková *et al.* 2006, 2009a).

Calculation methods

The programme Molinspiration Property Engine (v 2007.04, http://www.molinspiration.com/cgi-bin/properties) was used for calculation of partition coefficients (log P). The energy of the highest occupied molecular orbital (ε(HOMO)) and spin density values were calculated using HyperChem 8.0 Evaluation, Hypercube Inc., http://www.hyper.com, 2007, as described previously (Račková *et al.* 2005).

Statistical analyses

All values are given as means of 3–6 experiments ± SEM. Statistical significance of differences between means was established by Student´s t-test and one-way analysis of variance (ANOVA). *p*-values below 0.05 were considered statistically significant.

Tab. 1. IC_{50} doses of HHC and HHDC producing 50% inhibition of control chemiluminescence of whole blood.

	HHC	HHDC
	EC_{50} (μmol/l)	
OpZ	15.7 ± 2.2	>100
A23187	36.3 ± 14.9	>100
PMA	0.4 ± 0.1	2.4 ± 0.7

Percentage inhibition was calculated on the basis of integrated values of chemiluminescence over 1 800 s (A23187) or 3 600 s (OpZ, PMA). Control values, given in RLU × seconds were: 40 484 ± 2 177 (A23187), 162 088 ± 14 975 (OpZ) and 1 513 113 ± 198 401 (PMA).

Tab. 2. Electronic, hydrophobicity and antiradical activity values of HHC and HHDC.

Parameters	HHC	HHDC
HOMO energy, eV	-8.67	-9.16
Spin density at O. Atoms	-O.(7) 0.086 -O.(4´) 0.106	-O.(7) 0.156 -O.(4´) 0.158
logP	2.755	2.358
RM	0.52 ± 0.02	0.31 ± 0.02
*Reactivity with DPPH., s-1	0.055 ± 0.001	0.042 ± 0.001
*Reactivity with ABTS+., s-1	0.074 ± 0.001	0.064 ± 0.003

*˘- ΔA/ Δt, initial rate of absorbance decreases of radical solutions

RESULTS

Both HHC and HHDC significantly decreased CL of whole blood stimulated with receptor bypassing stimulus – PMA (0.05 μmol/l) from the concentration of 1 μmol/l (Figure 2A).

As shown in Table1, the effect of HHC is more intensive (IC_{50}: 0.4 ± 0.1 μmol/l) than that of HHDC (IC_{50}: 2.4 ± 0.7 μmol/l). Differences we detected also after OZ and A23187 stimulation. While HHC inhibited CL stimulated by A 23187 and OpZ, (IC50: 15.7 ± 2.2 and 36.3 ± 14.9 μmol/l, respectively), HHDC was ineffective (Figure 2A, B, C, Table 1).

The effects of HHC and HHDC on NO production by RAW 264.7 macrophages stimulated with LPS are demonstrated in Figure 3. HHC decreased NO production in the concentration of 10 μmol/l to 7.3 ± 1.3% of control and in the concentration of 100 μmol/l it completely inhibited NO production. On the other hand, HHDC was ineffective.

Table 2 shows electronic, hydrophobicity and antiradical activity values of HHC and HHDC. HHC was found to possess higher antiradical reactivity toward DPPH radical and toward ABTS·+ radical than HHDC, and showed higher values of hydrophobicity parameters (log P = 2.76, R_M = 0.52) compared to HHDC

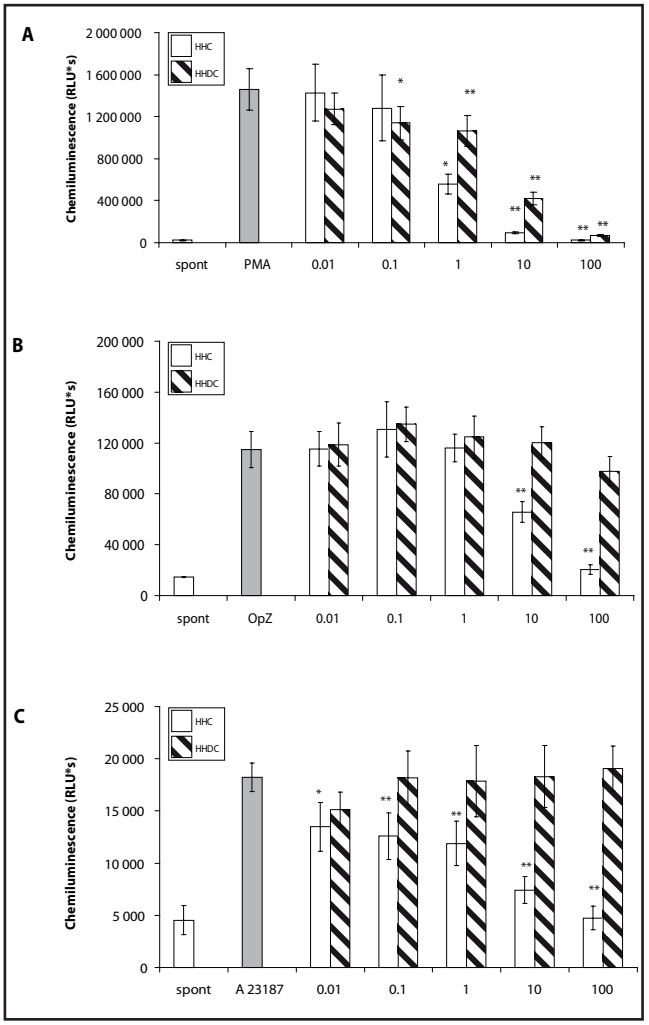

Figure 2 Effect of HHC and HHDC on stimulated whole blood chemiluminescence. Chemiluminescence was stimulated with PMA (0.05 µmol/l) **A**, OpZ (0.5 mg/ml) **B** or A23187 (1 µmol/l) **C**. spont = spontaneous (unstimulated chemiluminescence). The values represent the mean from 6 subjects ± SEM. **p<0.01, *p<0.05 as compared with the control in the absence of the substances tested.

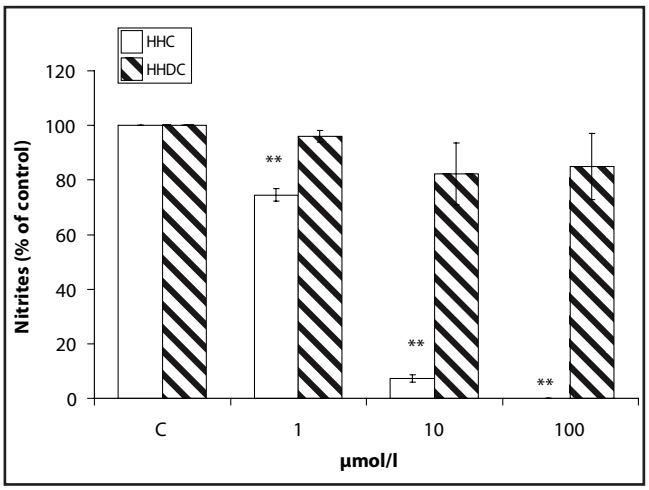

Figure 3 Effect of HHC and HHDC on LPS – stimulated nitrite production by macrophages. Data are expressed as mean±SEM of at least 3 independent experiments. **p<0.01 as compared with the control in the absence of the substances tested.

(logP = 2.36, R_M = 0.31), as well as a higher value of parameter ε(HOMO) (–8.67eV vs. –9.16eV). Furthermore, for HHC compared to HHDC, lower values of spin densities corresponding to O atoms of phenoxyl radicals derived from both alternative OH groups were found (Table 2).

DISCUSSION

In this study we present the effect of two synthetic phenycoumarin derivatives: HHC and HHDC on ROS generation in human neutrophils and on NO production by RAW 264.7 macrophages with respect to some of their physico-chemical parameters and intrinsic antiradical activity.

For neutrophil activation, we used three stimuli activating NADPH-oxidase in different ways: PMA via protein kinase C (PKC), OpZ after its binding to plasma membrane FcγR receptors, and A23187 by increasing the intracellular calcium level. Our results demonstrated an inhibitory effect of HHC on CL of human whole blood stimulated by either of three stimuli. The IC_{50} values showed a more intensive effect for PMA than for OpZ and A23187 stimulation (Table1). On the other hand, HHDC significantly inhibited only PMA stimulated CL. Since PMA stimulates ROS generation by direct activation of PKC, we suggest the interaction of HHC and HHDC to occur at PKC level. Recently we reported on the inhibitory effect of the natural substance diferuloylmethane (curcumin) on PKC, as indicated by decreased phosphorylation of PKC isoenzymes α and βII on their catalytic region (Jančinová *et al.* 2009a;b). Moreover, by docking simulation studies into protein kinase C (PKC) we showed that polyphenols involving flavonoids and coumarins might be efficient inhibitors of PKC, accounting thus for their indirect suppressive effect on ROS generation (Račková *et al.* 2009b). The structure-dependence of the inhibitory effect of polyphenolic antioxidants on signal transduction enzymes, such as PKC, was supported also by Ursini *et al.* (1994) and Varga *et al.* (2006).

Coumarins consist of a fused benzene and α-pyrone ring, which is an important group of low molecular phenolics, with a close structural relation to flavonoids. As substitutions can occur at any of the six available sites of their basic molecular moiety (1,2-benzopyrone), these compounds are extremely variable in their structure (Beillerot *et al.* 2008; Kostova 2005). HHC and HHDC have a similar structure. Both compounds differ only in the presence (or absence) of an unsaturated bound between C3 and C4 at ring C. The presence of double bond (in HHC) is forming a direct aromatic conjugation between the rings A and B, typical for stilbenoids, or for coumarin-stilbene hybrids (Vilar *et al.* 2006). The stilbene moiety in such structures might be responsible for the differences in the activity of the compounds (Quezada *et al.* 2010). Compound containing unconjugated dihydrostilbene moiety (as present in HHDC)

were in regulating NO production generally inactive (Harmatha *et al.* 2008).

Our results indicate that the inhibitory effect of HHC and HHDC on PMA stimulated ROS generation in neutrophils and on activation of PKC is probably not dependent on the presence of a double bound between C3 and C4 at ring C. On the other hand, only HHC reduced ROS production after OpZ and A 23187 stimulation, indicating the requirement of stilbene moiety formed by the 3,4-unsaturated bond at ring C for this inhibitory effect.

A further factor which probably plays a role in decreasing the CL by HHC is some component of the chemiluminescence system. The luminol reaction is highly dependent on the participation of myeloperoxidase, thus the reduction of the CL signal by HHC might be the result of decreased availability of peroxidase, due either to its decreased activity or liberation from azurophilic granules of neutrophils. The possibility of interaction with peroxidase is supported by findings of Kabeya *et al.* 2007 who demonstrated the inhibitory effect of 3-phenylcoumarin hydroxylated derivatives on horse radish peroxidase catalytic activity.

In RAW 264.7 macrophages, coumarinic derivatives were shown to inhibit LPS-stimulated release of nitric oxide, interleukin (IL)-1β, IL-6, prostaglandin E_2, and tumour necrosis factor (TNF) via the suppression of NF-κB activation (Bissonnette & Tremblay 2009). It has been suggested that the benzo-α-pyrone frame in the structure of coumarins is essential for anti-iNOS activity and suppression of iNOS expression might contribute to the reduction of NO production (Nakamura *et al.* 2009a,b), similarly as it was described for stilbene frame containing molecules (Harmatha *et al.* 2008), or for free stilbenoids of resveratrol type itself (Šmidrkal *et al.* 2010). In our experiments we detected a dose dependent inhibitory effect of HHC on NO production by RAW 264.7 macrophages stimulated with LPS, while HHDC did not exert any significant effect. Thus, similarly as mentioned above, the unsaturated 3,4-double bond at ring C is an important determinant in the inhibitory effect of HHC.

The dependence of radical scavenging activities of polyphenolic antioxidants on the number and positions of hydroxyl groups and other substituents on the molecule have been extensively studied (Lin *et al.* 2008, Račková *et al.* 2007, Riveiro *et al.* 2008). The antioxidant activity of polyphenols was reported to be due to their high reactivity as hydrogen or electron donors, to their capacity to chelate transition metal ions, and to the ability of polyphenolic radicals to stabilise and delocalise the unpaired electron (Riveiro *et al.* 2008). In accordance with these findings, we tested free radical-scavenging properties of HHC and HHDC, which might be involved in the decrease of ROS and NO production by phagocytes. The conjugated aromatic system in HHC may account for its better electron releasing capacity and more proper stabilisation of the phenoxyl radicals, generated in the course of reaction with oxidants, compared to the system of aromatic rings in HHDC isolated through double-bond saturation. In consistency with this assumption, we observed a higher free radical reducing efficacy of HHC compared to HHDC. This was in accordance with the calculated higher value of parameter ε(HOMO) for HHC, reflecting electron donor properties of the molecule, and with its lower values of spin densities at oxygen atoms corresponding to the phenoxyl radicals derived from the 4´ and 7 OH groups, suggesting a more efficient stabilisation of the phenoxyl radicals generated from HHC (Table 2). Moreover, the higher values of lipophilicity parameters of HHC suggest a more efficient partitioning of HHC into the membrane compared to the less lipophilic HHDC, which can further favour the better biological efficacies of HHC in the cellular system.

In conclusion, the present work provides new information on the action of two synthetic phenylcoumarin derivatives, HHC and HHDC, on ROS generation in human neutrophils and on NO production by RAW 264.7 macrophages. The obtained results highlighted the different effects of HHC and HHDC on phagocyte functions, which might be due to their diverse free radical scavenging properties and lipophilicity features.

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