The Influence of some Xanthone Derivatives on the Activity of J-774A.1 Cells

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Abstract
The chemiluminescence of stimulated cells with phorbol myristate acetate and the production of nitric oxide after stimulation with lipopolisaccharide in the presence of the parent compounds FAA (flavone-8-acetic acid = (4-oxo-2-phenyl-4H-chromen-8-yl)acetic acid), XAA (xanthone-4-acetic acid = (9-oxo-9H-xanthen-4-yl)acetic acid), and appropriate xanthone derivatives (1–7) was determined. Also the toxicity of the FAA, MFAA ((6-methyl-4-oxo-2-aryl-4H-chromen-8-yl)acetic acid), XAA and 1–7 against J-774A.1 cultured cells was evaluated. Compound 5 (2-methyl-2-[(9-oxo-9H-xanthen-2-yl)methyl]sulfanyl)-propanoic acid) was effective in inhibiting chemiluminescence of J-774A.1 cells but most of the other tested compounds stimulated the reaction. FAA and two xanthones with a methoxycarbonyl moiety slightly decreased the generation of nitric oxide at 50 μM. Most of the tested compounds (1–7) showed weak toxicity at concentration of 100 μM.

Keywords
Xanthone derivatives • J-774A.1 cells • Chemiluminescence • Toxicity
Introduction

Among the different classes of antitumor agents, the some flavone and xanthone derivatives are an important group of compounds with anticancer activity [1, 2]. Earlier studies have shown the advantageous properties of synthetic flavone-8-acetic acid (FAA, NSC 347512; (4-oxo-2-phenyl-4H-chromen-8-yl)acetic acid) [3] i.e. against advanced experimental colon tumors in mice [4]. Additionally, FAA has a different toxicity profile to most anticancer drugs, with no significant myelosuppression observed [5]. The related compounds MFAA (6-methyl-4-oxo-2-aryl-4H-chromen-8-yl)acetic acid) containing a 6-methyl substituent in FAA showed antitumor activity comparable to FAA in vitro but were essentially inactive in vivo [6]. Also the closely related class of compounds FAA and MFAA the xanthone-4-acetic acid ((9-oxo-9H-xanthen-4-yl)acetic acid; XAA) [7] and some of its derivatives, in particular 5,6-dimethylxanthone-4-acetic acid ((5,6-dimethyl-9-oxo-9H-xanthen-4-yl)acetic acid; DMXAA) [8, 9] showed promising antitumor activity. A recent comparative study of FAA and DMXAA, with a series of derivatives of XAA, in which the substituents in position 5 and 6 were included in five or six-membered rings suggest cytotoxicity activity in a preliminary in vitro assay, comparable to the parent compounds [10].

![Sch. 1.

Earlier studies have shown that functions of activated macrophages such as killing of tumor cells, release of cytokines and generation of oxygen radicals can be regulated by flavone [11, 12]. They have also been shown to inhibit oxido–reductases [13], thus preventing the formation of free radicals resulting from the reduction of oxygen. FAA and analogues increases the direct cytotoxicity of murine macrophages in vitro against tumor targets [14], and stimulates the formation of nitric oxide [15]. Nitric oxide (NO) is one from a variety of mediators released by activated macrophages. It has been identified as potent molecule that may exert regulatory or cytotoxic effects depending on the concentration acting on the target cell [16, 17]. The results of our earlier studies [18] suggest that the flavones can modulate the immune responses and the inflammatory reactions by controlling production of nitric oxide. J-774A.1 cells are functional similar to murine macrophages [19, 20].

In this study the chemiluminescence of stimulated cells with phorbol myristate acetate (PMA) and the production of nitric oxide after stimulation with lipopolysaccharide (LPS) in the presence of the parent compounds FAA, XAA and xanthone derivatives 1–7 were determined.
Results and Discussion

Chemistry

FAA was derived from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, USA, through the courtesy of Dr. Paull. Other xanthone derivatives (XAA and 1-5) were synthesized as previously described [7, 21–23]. Compound 6 was obtained by condensation of 2-methyl-6-hydroxyxanthone [24] with α-bromopropionic acid according to published procedure for 4 [23]. Compound 7 was readily formylated according to Link [25], using 2-methyl-6-hydroxyxanthone, solid sodium hydroxide, acetone and chloroform. Formation of appropriate analogues of clofibrate under Link conditions is a widely used reaction [26,27]. Some of them (XAA, 2, 6 and 7) were characterized by formation of appropriate esters (XAAa, 2a, 6a and 7a) (Tab.1), according to well known procedures.

Tab. 1. Structure of the synthesized compounds

<table>
<thead>
<tr>
<th>Cpd</th>
<th>R&lt;sup&gt;1&lt;/sup&gt;</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>XAA</td>
<td>4-CH&lt;sub&gt;2&lt;/sub&gt;-COOH</td>
<td>H</td>
</tr>
<tr>
<td>XAAa</td>
<td>4-CH&lt;sub&gt;2&lt;/sub&gt;-COOMe</td>
<td>H</td>
</tr>
<tr>
<td>1</td>
<td>2-CH&lt;sub&gt;2&lt;/sub&gt;-COOH</td>
<td>H</td>
</tr>
<tr>
<td>2</td>
<td>2-CH&lt;sub&gt;2&lt;/sub&gt;-COOH</td>
<td>6-OMe</td>
</tr>
<tr>
<td>2a</td>
<td>2-CH&lt;sub&gt;2&lt;/sub&gt;-COOMe</td>
<td>6-OMe</td>
</tr>
<tr>
<td>3</td>
<td>2-CH&lt;sub&gt;2&lt;/sub&gt;-COOH</td>
<td>7-Cl</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>H</td>
</tr>
<tr>
<td>5</td>
<td>2-Me COOH</td>
<td>H</td>
</tr>
<tr>
<td>6</td>
<td>2-Me COOH</td>
<td>2-Me</td>
</tr>
<tr>
<td>6a</td>
<td>2-Me COOEt</td>
<td>2-Me</td>
</tr>
<tr>
<td>7</td>
<td>2-Me COOH</td>
<td>2-Me</td>
</tr>
<tr>
<td>7a</td>
<td>2-Me COOMe</td>
<td>2-Me</td>
</tr>
</tbody>
</table>

Pharmacology

The studied compounds can influence on cell viability. Macrophages, as phagocytes during activation, are dynamic cells that characterize movement of cell membrane. To preliminary study of cytotoxic effect of tested compounds during experiments it was used method based on measurements of LDH leakage from the cells. Neither of the tested xanthone derivatives at concentration 50 µM showed toxicity against J7774A.1 cells cultured for 24h, but at concentration 100 µM most of them showed weak toxicity (Tab. 2).
Tab. 2. Effects of tested compounds on cell viability (cytotoxic effect) determined with LDH method

<table>
<thead>
<tr>
<th>Cpds</th>
<th>Cytotoxic effect at concentration 100µM [%] Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>FAA</td>
<td>0</td>
</tr>
<tr>
<td>XAA</td>
<td>5.0±1.0</td>
</tr>
<tr>
<td>1</td>
<td>1.0±0.6</td>
</tr>
<tr>
<td>2</td>
<td>1.2±0.7</td>
</tr>
<tr>
<td>3</td>
<td>3.9±0.8</td>
</tr>
<tr>
<td>4</td>
<td>8.2±2.6</td>
</tr>
<tr>
<td>5</td>
<td>7.5±1.8</td>
</tr>
<tr>
<td>6</td>
<td>3.9±1.0</td>
</tr>
<tr>
<td>7</td>
<td>6.3±1.5</td>
</tr>
</tbody>
</table>

Percentages of cytotoxicity at concentration 50µM of tested compounds were equal zero. The cells were cultured with compounds for 24h. The data are the mean values from three experiments.

Stimulation of macrophages by PMA induces process, named respiratory burst, mainly combined with generation of reactive forms of oxygen. The process remember phagocytosis when the cells destroying microorganisms. The phagocytosis is important in defense system against invading microbial pathogens. On the other hand overproduction of the oxygen metabolites may induce pathologies [36–38].

Tab. 3. Effects of tested compounds on chemiluminescence and generation of nitrite

<table>
<thead>
<tr>
<th>Cpds</th>
<th>Chemiluminescence [% control] Mean±SEM</th>
<th>Nitrite concentration [µM] Mean±SEM</th>
<th>Nitrite concentration after stimulation with LPS [µM] Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P&lt;</td>
<td>P&lt;</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>5.7±1.0</td>
<td>25.7±0.4</td>
</tr>
<tr>
<td>FAA</td>
<td>137±8.7</td>
<td>0.05a</td>
<td>5.7±0.9</td>
</tr>
<tr>
<td>XAA</td>
<td>127±8.1</td>
<td>0.05</td>
<td>5.8±0.8</td>
</tr>
<tr>
<td>1</td>
<td>149±12.7</td>
<td>0.05</td>
<td>5.5±0.5</td>
</tr>
<tr>
<td>2</td>
<td>112±4.6</td>
<td>n.s.</td>
<td>5.8±0.2</td>
</tr>
<tr>
<td>3</td>
<td>114±13.3</td>
<td>n.s.</td>
<td>7.2±0.7</td>
</tr>
<tr>
<td>4</td>
<td>113±2.3</td>
<td>0.02</td>
<td>7.2±0.9</td>
</tr>
<tr>
<td>5</td>
<td>72±6.9</td>
<td>0.05</td>
<td>7.3±0.9</td>
</tr>
<tr>
<td>6</td>
<td>117±5.8</td>
<td>n.s.</td>
<td>7.2±0.5</td>
</tr>
<tr>
<td>7</td>
<td>131±4.6</td>
<td>0.005</td>
<td>5.5±0.4</td>
</tr>
</tbody>
</table>

*a Level of significance of t-Student’s test. Values are compared to control (without tested compounds). n.s. – no significance. Concentration of tested compounds – 50µM. The data are the mean values from three experiments.
Chemiluminescence (Tab. 3) was only inhibited in the presence of compound 5 with the 2-thio-2-methyl propionic moiety. Other of tested compounds increased or did not change chemiluminescence. In study [12] FAA and xanthone acetic analogues these compounds changed PMA stimulated generation of superoxide anion by murine macrophages dependent on concentration.

Compounds at lower concentration stimulated but at higher concentration decreased generation of superoxide anion. Early studies with hydroxy and methoxyxanthone derivatives [28] showed stimulation of protein kinase C (PKC) isoforms. PMA induces respiratory burst via PKC [29]. This mechanism may be in the presence of tested compounds. Compounds XAA and 1 (different position of the methyl carboxyl group) showed similar activity. Stimulation the J-774A.1 cells, as a model cells, by LPS induces generation of nitric oxide radical. The strong stimulation of macrophages in the body by pathogens generating a big amount of nitric oxygen and many other reactive substances destroying an environmental of the body. The activity of nitric oxide is dependent on place and concentration and this process is very difficult to control [39–41]. Tested compounds did not change generation of nitrite by unstimulated cells and slightly decreased generation of nitrite by stimulated J-774A.1 cells with LPS. The most activity in decreasing of nitrite generation was observed in the presence of FAA and xanthone derivatives with methyl carboxyl group. This result is in opposition to data by Thomsen [30]. That study was performed with FAA at higher concentration. Only 5,6-dimethyl-XAA was more active at low concentration. In our study concentration of tested compounds was lower because of cytotoxic effect.

The tested compounds in our study have not hydroxyl substituents at the main xanthone structure. The hydroxyxanthone derivatives, tested by other authors, showed an inhibitory effect on stimulated macrophages [31, 32]. It is probably dependent on an activity of these groups to reaction with oxygen and nitrogen intermediates.

The chosen most active xanthone derivatives, after additional studies, may be use as a modulator of process accompanying with reactions of free radicals.

Further studies on the biological effects of the active xanthone derivatives are in progress.

**Experimental**

**Chemistry**

$^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker spectrometer 500.13 MHz, using signal from DMSO in DMSO-d$_6$ and TMS in CDCl$_3$ as internal standards. MS were recorded using an AMD-604 mass spectrometer (70eV). The IR spectra were recorded on a Perkin Elmer or Jasco FT/IR 410 spectrometer (KBr pellets). M.p.s are uncorrected and were determined using a Büchi SMP-20 apparatus. Microanalyses were performed in the Department of Pharmaceutical Chemistry of Jagiellonian University, Medical College. All the results were within an acceptable range.

Methyl (9-oxo-9H-xanthen-4-yl)acetate (XAAa)

Yield: 76%, m.p. 155–157°C (methanol). IR (cm\(^{-1}\)): 2950, 1732, 1655, 1616, 1603, 1446, 1347, 1226, 1215, 1172. \(^1\)H NMR (500.13 MHz) (\(\delta\), ppm, DMSO-d\(_6\)): 8.21 (1H, dd, J = 7.9 Hz, J = 1.7 Hz, H-8), 8.14 (1H, dd, J = 7.9 Hz, J = 1.7 Hz, H-1), 7.90 (1H, ddd, J = 8.5 Hz, J = 7.1 Hz, J = 1.7 Hz, H-6), 7.84 (1H, dd, J = 7.2 Hz, J = 1.7 Hz, H-3), 7.63 (1H, dd, J = 8.5 Hz, J = 1.0 Hz, H-5), 7.51 (1H, ddd, J = 7.9 Hz, J = 1.0 Hz, H-7), 7.46 (1H, dd, J = 7.9 Hz, J = 7.2 Hz, H-2), 4.09 (2H, s, CH\(_2\)). \(^13\)C NMR (500.13 MHz) (\(\delta\), ppm, DMSO-d\(_6\)): 175.92 (C=O), 170.85 (COO), 155.17 (C-4b), 153.76 (C-4a), 136.79 (C-3), 135.56 (C-6), 125.90 (C-8), 124.96 (C-1), 124.51 (C-7), 124.33 (C-4), 123.83 (C-2), 121.02 (C-8b), 120.81 (C-8a), 118.10 (C-5), 51.86 (OCH\(_3\)), 34.49 (Ar-CH\(_2\)). C\(_{16}H\(_{12}\)O\(_4\) (268.3).

Methyl (6-methoxy-9-oxo-9H-xanthen-2-yl)acetate (2a)

Yield: 73%, m.p. 159–160°C (methanol). IR (cm\(^{-1}\)): 2950, 1734, 1654, 1619, 1576, 1453, 1256, 1162. \(^1\)H NMR (500.13 MHz) (\(\delta\), ppm, DMSO-d\(_6\)): 8.09 (1H, d, J = 8.8 Hz, H-8), 8.07 (1H, dd, J = 2.3 Hz, J = 0.5 Hz, H-1), 7.74 (1H, dd, J = 8.5 Hz, J = 2.4 Hz, H-4), 7.14 (1H, d, J = 2.4 Hz, H-5), 7.04 (1H, dd, J = 8.8 Hz, J = 2.4 Hz, H-7), 3.93 (3H, s, CH\(_3\)OAr), 3.89 (2H, s, CH\(_2\)), 3.65 (3H, s, CH\(_3\)). \(^13\)C NMR (500.13 MHz) (\(\delta\), ppm, DMSO-d\(_6\)): 174.73 (C=O), 171.38 (COO), 164.89 (C-6), 157.48 (C-4b), 154.55 (C-4a), 136.21 (C-3), 130.58 (C-2), 127.51 (C-8), 126.27 (C-1), 120.87 (C-8b), 117.82 (C-4), 114.84 (C-8a), 113.54 (C-7), 100.52 (C-5), 56.07 (CH\(_3\)OAr), 51.71 (OCH\(_3\)), 39.05 (CH\(_2\)). C\(_{17}H\(_{14}\)O\(_5\) (298.3).

(\(\pm\))-2-[(7-Methyl-9-oxo-9H-xanthen-3-yl)oxy]propanoic acid (6)

Yield: 63%, m.p. 201–203°C (ethanol). IR (cm\(^{-1}\)): 3438, 2922, 1749, 1655, 1618, 1578, 1481, 1310, 1257, 1234, 1206, 1177, 1113. \(^1\)H NMR (500.13 MHz) (\(\delta\), ppm, DMSO-d\(_6\)): 13.30 (1H, bs, CO\(_2\)H), 8.10 (1H, dd, J = 8.7 Hz, J = 0.5 Hz, H-8), 7.94 (1H, ddd, J = 2.3 Hz, J = 0.5 Hz, H-1), 7.65 (1H, ddd, J = 8.5 Hz, J = 2.3 Hz, J = 0.6 Hz, H-3), 7.53 (1H, ddd, J = 8.5 Hz, J = 0.5 Hz, J = 0.5 Hz, H-4), 7.03 (1H, dd, J = 8.7 Hz, J = 2.3 Hz, J = 0.5 Hz, H-7), 7.01 (1H, dd, J = 2.3 Hz, J = 0.5 Hz, H-5), 5.13 (1H, q, J = 6.8 Hz, CH), 2.43 (3H, d, J = 0.6 Hz, CH\(_3\)), 1.58 (3H, d, J = 6.8 Hz, CH\(_3\)). \(^13\)C NMR (500.13 MHz) (\(\delta\), ppm, DMSO-d\(_6\)): 174.77 (C=O), 172.16 (COOH), 162.91 (C-6), 157.17 (C-4b), 153.79 (C-4a), 135.98 (C-3), 133.66 (C-2), 127.64 (C-8), 125.10 (C-1), 120.78 (C-8b), 117.67 (C-4), 115.19 (C-8a), 113.71 (C-7), 101.46 (C-5), 72.15 (CH\(_2\)OAr), 20.25 (CH\(_3\)-Ar), 17.91 (CH\(_3\)-CH). MS (m/z): 298 (M\(^+\)), 253 (100%), 226, 209, 197, 181, 169, 153, 126, 115. C\(_{17}H\(_{14}\)O\(_5\)) (298.3).

(\(\pm\))-Ethyl 2-[(7-methyl-9-oxo-9H-xanthen-3-yl)oxy]propanoate (6a)

Yield: 71%, m.p. 121–123°C (ethanol). IR (cm\(^{-1}\)): 2991, 2932, 1732, 1656, 1614, 1591, 1480, 1444, 1305, 1289, 1252, 1232, 1175, 1107. \(^1\)H NMR (500.13 MHz) (\(\delta\), ppm, DMSO-d\(_6\)): 8.10 (1H, dd, J = 8.7 Hz, J = 0.5 Hz, H-8), 7.95 (1H, ddd, J = 2.3 Hz, J = 0.9 Hz, J = 0.5 Hz, H-1), 7.66 (1H, ddd, J = 8.6 Hz, J = 2.3 Hz, J = 0.6 Hz, H-3), 7.53 (1H, dd, J = 8.6 Hz, J = 0.5 Hz, H-4), 7.06 (1H, dd, J = 2.4 Hz, J = 0.5 Hz, H-5), 7.04 (1H, dd, J = 8.7 Hz, J = 2.4 Hz, H-7), 5.28 (1H, q, J = 6.8 Hz, CH), 4.19 (2H, dq, J = 10.9 Hz, J = 7.1 Hz, CHH(Et)), 4.17 (2H, dq, J = 10.9 Hz, J = 7.1 Hz, CHH(Et)), 2.44 (3H, dd, J = 0.9 Hz, J = 0.5 Hz, J = 8.6 Hz, CH\(_3\)), 1.59 (3H, d, J = 6.8 Hz, CH\(_3\)), 1.20 (3H, t, J = 7.1 Hz, CH\(_3\)). \(^13\)C NMR (500.13 MHz) (\(\delta\), ppm, DMSO-d\(_6\)): 174.79 (C=O), 170.58 (COOH), 162.67 (C-6), 157.18.
The Influence of some Xanthone Derivatives on the Activity of J-774A.1 Cells

2-Methyl-2-[(7-methyl-9-oxo-9H-xanthen-3-yl)oxy]propanoic acid (7)
Yield: 62%, m.p. 212–214°C (ethanol). IR (cm⁻¹): 3436, 2941, 1743, 1654, 1603, 1575, 1448, 1250, 1140, 1108.¹H NMR (500.13 MHz) (δ, ppm, DMSO-d₆): 13.40 (1H, s, CO₂H), 8.09 (1H, d, J = 8.9 Hz, H-8), 7.92 (1H, d, J = 2.3 Hz, H-1), 7.61 (1H, ddd, J = 8.4 Hz, J = 2.3 Hz, J = 0.6 Hz, H-3), 7.50 (1H, d, J = 8.5 Hz, H-4), 6.95 (1H, dd, J = 8.9 Hz, J = 2.4 Hz, H-7), 6.82 (1H, d, J = 2.4 Hz, H-5), 2.42 (3H, s, CH₃Ar), 1.67 (6H, s, 2xCH₃). ¹³C NMR (500.13 MHz) (δ, ppm, DMSO-d₆): 175.99 (CO), 175.36 (COOH), 162.16 (C-6), 157.83 (C-4b), 154.82 (C-4a), 137.11 (C-3), 134.77 (C-2), 128.60 (C-8), 126.11 (C-1), 121.73 (C-8b), 118.75 (C-4), 116.62 (C-7), 116.18 (C-8a), 104.74 (C-5), 80.73 (C-O-Ar), 26.03 (2x CH₃), 21.34 (CH₃-Ar). MS (m/z): 312 (M⁺), 267, 226 (100%), 197, 181, 169, 115. C₁₉H₁₈O₅ (326.3).

Methyl 2-methyl-2-[(7-methyl-9-oxo-9H-xanthen-3-yl)oxy]propanoate (7a)
Yield: 74%, m.p. 120–122°C (methanol). IR (cm⁻¹): 2993, 2956, 1737, 1651, 1620, 1593, 1482, 1445, 1301, 1261, 1172.¹H NMR (500.13 MHz) (δ, ppm, DMSO-d₆): 8.09 (1H, d, J = 8.9 Hz, H-8), 7.93 (1H, d, J = 2.3 Hz, H-1), 7.63 (1H, ddd, J = 8.5 Hz, J = 2.3 Hz, J = 0.7 Hz, H-3), 7.50 (1H, d, J = 8.5 Hz, H-4), 6.91 (1H, dd, J = 8.8 Hz, J = 2.4 Hz, H-7), 6.81 (1H, d, J = 2.4 Hz, H-5), 3.76 (3H, s, CH₃O), 2.43 (3H, s, CH₃Ar), 1.68 (6H, s, 2xCH₃). ¹³C NMR (500.13 MHz) (δ, ppm, DMSO-d₆): 176.01 (CO), 174.10 (COOH), 161.79 (C-6), 157.84 (C-4b), 154.86 (C-4a), 137.24 (C-3), 134.84 (C-2), 128.73 (C-8), 126.13 (C-1), 121.73 (C-8b), 118.82 (C-4), 116.55 (C-7), 116.48 (C-8a), 105.27 (C-5), 80.75 (C-O-Ar), 58.81 (OCH₃), 26.00 (2x CH₃), 21.31 (CH₃-Ar). MS (m/z): 312 (M⁺), 267, 226 (100%), 197, 181, 169, 115. C₁₉H₁₆O₅ (312.3).

Pharmacology
Compounds were dissolved in RPMI 1640 medium without phenol red (Gibco, Grand Island, NY) or in Hanks buffer (HBSS) pH 7.4 without phenol red (Gibco, Grand Island, NY).

J-774A.1 cells
Murine J-774A.1 macrophages were obtained from German Collection of Microorganisms and Cell Cultures, Dept. Human and Animal Cell Cultures in Braunschweig. During the study cells were grown in RPMI 1640 medium without phenol red (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (South American Origin, BIO WHITTAKER EUROPE, Verviers, Belgium), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C and 5% CO₂ atmosphere.

During culture and experiments cell viability was controlled by lactate dehydrogenase method (Cytotoxicity Detection Kit (LDH)–Roche Diagnostics GmbH, Mannheim, Germany).

Cell stimulation
The adherent cells were incubated with tested compounds (0.5 h) and next stimulated with...
lipopolisaccharide (100 ng/mL) from *E. coli* (0127:B8) (Calbiochem, La Jolla, CA, USA) After 24h culture of the cells the supernatants were removed and assayed for levels of nitrite.

**Nitrite assay**

Nitrite concentration in the culture medium was measured by a microplate assay method, based on the Griess reaction [33]. Equal volumes of culture medium supernatant and Griess reagent (0.5% sulfanilamide, 0.05% naphthylene-diamide dihydrochloride in 2.5% H$_3$PO$_4$) were added to microplate and incubated at 25°C for 10 min. The absorbance of culture medium and Griess reagent at 550nm was determined with Microplate Reader ELX 800 (Bio-tek Instruments, Inc., Winooski, VT, USA).

**Chemiluminescence**

Into 10$^6$ cells with tested compounds or with HBSS (controls), luminol (Sigma, St. Louis, MO, USA) solution was added, giving a final concentration 0.1 mM and 5 min later phorbol myristate acetate (PMA) (Sigma, St. Louis, MO, USA) solution (final concentration 0.8 μM) as stimulus was used. The final volume of each sample was 1 mL. Chemiluminescence (CL) was measured for 20 min (5 min with luminol and 15 min, after that, PMA was added) using a system equipped with a photomultiplier 9514s from THORN EMI (Middlesex, England) [34, 35]. The intensity of CL was determined by measuring counts/min and by calculating the area under the CL curve. Then the percentages of control were calculated.

**Authors’ Statement**

**Competing Interests**

The authors declare no conflict of interest.

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