Effects of quinoline-arylamidine hybrids on LPS-induced inflammation in RAW 264.7 cells

Abstract

Background and purpose: Inflammation is a common pathogenesis in infection, injury, cancer, and many chronic diseases. Macrophages are among the main cells involved in generation of inflammation. The aim of the present study was to investigate the effects of molecular hybrids with 7-chloroquinoline and arylamidine moieties joined through flexible 2-aminoethanol linker, on the in vitro inflammatory responses to lipopolysaccharides (LPS) induced inflammation in the RAW 264.7 cells.

Materials and methods: To determine effects of seven quinoline-arylamidine hybrids on the growth of the murine macrophage-like (RAW 264.7) cells MTT assay was used. Inflammatory reactions in the RAW264.7 cells were induced using E. coli lipopolysaccharides (LPS). Levels of nitric oxide (NO) and malondialdehyde (MDA) were determined by spectrophotometry methods. Intracellular production of reactive oxygen species (ROS) was measured by flow cytometry. Antioxidant capacity of tested compounds was tested by 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical cation method.

Results: Tested hybrid compounds differentially influenced proliferation of non-stimulated and LPS-stimulated RAW 264.7 cells. The hybrid compounds have not presented ABTS radical-scavenger activity. In the LPS-stimulated RAW 264.7 cells 10 μM compounds slightly decreased production of NO and ROS and significantly modulated LPS-induced lipid peroxidation.

Conclusions: Molecular hybrids with 7-chloroquinoline and arylamidine moieties joined through flexible 2-aminoethanol linker markedly decreased accumulation of lipid peroxidation products in the LPS-stimulated RAW 264.7 cells. Further studies are necessary to determine their mechanism of anti-inflammatory action in more details.

INTRODUCTION

Inflammation is a common pathophysiological process in infection, injury, cancer, and many chronic diseases including autoimmune diseases, diabetes, Alzheimer’s disease, cardiovascular and inflammatory bowel diseases (1). Although in the last two decades anti-inflammatory therapy in chronic diseases triggered by inflammation, deregulation or autoimmunity has significantly improved, there are considerable limitations while treatment choices of inflammatory process often express side effects or/and lack of effectiveness. For that reason, there is a continuing need to develop potent and less harmful anti-inflammatory drugs (2).
Nitrogen-containing heterocyclic molecules are obligatory structural units in medicinal chemistry with diverse applications. Among them, quinoline and its derivatives have attracted considerable attention due to their various pharmacological activities including antitumor, antioxidant, antiproliferative (3), and anti-inflammatory activity (4, 5, 6). Having in mind that new drug development strategies are focusing on molecules that act simultaneously on multiple targets, either by a combination of at least two drugs or by combining two (or more) active pharmacophores in the single-hybrid molecule that has dual activity (7, 8), we recently reported on the synthesis and antitumor activity of four series of new molecular hybrids containing 7-chloroquinoline and arylamidine moieties joined through the rigid -O- or flexible -NH-CH₂-CH₂-O- linker (9).

We found that the new hybrid molecules with 7-chloroquinoline displayed selectivity on carcinoma and leukemia cells, showing higher efficacy against leukemia cells (10).

Since the causal relationship between inflammation, innate immunity and cancer is more widely accepted and cancer-related inflammation has been proposed to promote tumor progression and serve as the seventh hallmark of tumor, we decided to expand our investigation to test anti-inflammatory potential of selected hybrid compounds.

Macrophages are the cornerstone of the innate immune system, which activation plays a significant role in homeostasis of organisms (11). In vitro macrophage activation techniques provide us with a window to understand the mechanisms of inflammation and response of macrophages to the modulating interventions (12). Nitric oxide (NO) is an important pro-inflammatory mediator produced by inducible nitric oxide synthase (iNOS) during conversion of L-arginine to L-citrulline. The reaction of NO with superoxide anion will form a strong cytotoxic oxidant, peroxynitrite, which increases the production of PGs and causes lipid peroxidation and cellular damage (13). Pro-inflammatory cytokines are produced in large quantities by activated macrophages/monocytes that stimulate cellular responses via increasing prostaglandins (PGs) and reactive oxygen species (ROS). Additionally, lipid peroxidation (malondialdehyde, MDA) is produced by free radicals attacking the cell membranes. Thus, inflammatory effect results in the accumulation of MDA.

The aim of the present study was to investigate in vitro anti-inflammatory potential of molecular hybrids with 7-chloroquinoline and arylamidine moieties joined through flexible 2-aminoethanol linker. The anti-inflammatory activities of selected hybrids were evaluated by means of inhibiting reactive oxygen species (ROS) and nitric oxide (NO) production as well as MDA accumulation in the lipopolysaccharides (LPS) induced RAW 264.7 cells.

**MATERIAL AND METHODS**

**Compounds**

The synthesis, physical properties, and antitumor activity of new molecular hybrids with 7-chloroquinoline and arylamidine moieties joined through flexible 2-aminoethanol linker have been described previously (9). Structural details of all studied molecules are shown in Figure 1.

**Cell proliferation assay**

Effects of tested compounds on the murine macrophage-like cell line RAW264.7 (ATCC® TIB-71) viability were determined using the colorimetric methyltetrazolium (MTT) assay. Briefly, RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 10 mM sodium pyruvate and 2 mM HEPES. The cells were grown in tissue culture flasks (BD Falcon, Germany) in the humidified atmosphere under the conditions of 37°C/5% of CO₂ gas in the CO₂ incubator (IGO 150 CEllLife™, JOUAN, Thermo Fisher Scientific, Waltham, MA, USA). The cells were seeded...
in 96 micro-well plates at a concentration of 2×10⁴ cells/cm² and treated with hybrid compounds 1a–1g at a concentration range 100–0.1 μM for 72 h. Additionally, cells were pretreated with 10 μM 1a–1g or Trolox, as a reference compound, for 2 h and then stimulated with LPS (1μg/mL) for 24 h. After 24 and 72 h of incubation, medium was removed and 5 mg/cm² MTT solution (Merck, New Jersey, SAD) was added to each well and incubated for 4 h in the CO₂ incubator. Then, to each well, DMSO was added to dissolve water-insoluble MTT-formazane crystals. The Elisa microplate reader (iMark, BIO RAD, Hercules, CA, USA) was used for measurement of the absorbance at 595 nm. All experiments were performed at least three times in triplicates. The percentage of cell growth (PG) was calculated using the following equation:

\[ \text{PG} = \left( \frac{A_{\text{compound}} - A_{\text{background}}}{A_{\text{control}} - A_{\text{background}}} \right) \times 100 \]

where, \( A_{\text{background}} \) is the absorbance of MTT solution and DMSO; \( A_{\text{control}} \) is the absorbance of cell suspension grown without tested compounds.

For the purpose of calculating IC₅₀ values, cells were treated for 72 h with compounds at concentrations of 1, 10, 50 and 100 μM. Briefly, individual concentration effect curves were generated by plotting the logarithm of the concentration of tested compounds.

**Total antioxidant capacity assay**

Total antioxidant capacity of the compounds 1a–1g was measured according to the method described previously on the basis of cell quenching capacity toward the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical cation (14). ABTS⁺ was produced by the reaction between 7 mM water solution of ABTS (Sigma-Aldrich, St. Louis, MO, USA) and 2.45 mM ammonium persulfate (Sigma-Aldrich, St. Louis, MO, USA) (1:1), stored in the dark at room temperature for 12–16 h before the use. The ABTS⁺ solution was then diluted with methanol to obtain an absorbance of 0.700 at 750 nm. Adapted ABTS⁺ solution was mixed with 50 μM compounds (1:1). Esculetin and Trolox were used as standard compounds and were applied with the same concentration. Absorbance (A) was measured after 30 or 60 minutes of incubation in the dark at a room temperature after the initial mixing. An appropriate solvent blank was run in each assay. All the measurements were carried out at least three times. Percentage of inhibition of absorbance at 750 nm was calculated using the formula, ABTS⁺ scavenging effect (%) = 100 – [(A / A₀) × 100] where, \( A₀ \) is absorbance of ABTS radical + methanol, while \( A \) is absorbance of ABTS radical + tested hybrids/standard.

**Measurement of intracellular ROS by flow cytometry**

The intracellular ROS produced by non-stimulated (control) and LPS-stimulated macrophages was detected by the Fluorometric Intracellular ROS Kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer’s instructions. RAW264.7 cells (5×10⁵ cells/cm²) were plated in 24-well plates and left over night in CO₂ incubator. The non-stimulated RAW264.7 cells (5 × 10⁵ per well in a 24-well plate) were incubated for 2 h with 10 μM hybrid compounds or Trolox. Stimulated cells were pre-incubated with 10 μM compounds for 2 h and then continuously stimulated with 1 μg/mL LPS (Lipopolysaccharides from Escherichia coli O111:B4; Sigma-Aldrich, St. Louis, MO, USA) for 24 h. After the treatment cells were labeled with 1 μL of 500 x ROS detection reagent stock solution in 1 mL of cells suspension for 30 min at 37 °C in a humidified atmosphere at 5% CO₂. The fluorescence intensity was measured by flow cytometry (FacsCanto II, BD Biosciences, USA) and analyzed using FlowJo software (Inivai Technologies, USA).

**Measurement of nitrite concentration**

Nitrite production as an indicator of nitric oxide (NO) intracellular levels was measured in the supernatant of the RAW264.7 macrophages (15). Cells (5 × 10⁵ cells/cm²) were plated in 24-well plates and left over night in the CO₂ incubator. Stimulated cells were pre-incubated with 10 μM compounds for 2 h and then continuously stimulated with 1 μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Cell’s supernatant was collected and mixed in 1:1 ratio with Griess reagent (1% sulfanilamide in 5% phosphoric acid, and 0.1% naphthylethlenediamide, Sigma-Aldrich, St. Louis, MO, USA) dissolved in water. The mixture was then incubated at room temperature for 15 min in darkness. After 15-min incubation, the absorbance was read with an ELISA microplate reader (iMark, BIO RAD, Hercules, CA, USA). The nitrite concentrations were calculated from a sodium nitrite standard curve, and the NO concentrations in the supernatant were determined by comparison with the standard curve.

**Assessment of lipid peroxidation**

Lipid peroxidation was assayed by the measurement of the malondialdehyde (MDA) level by the thiobarbituric acid reacting substance (TBARS) method (16). RAW264.7 cells (5 × 10⁵ cell/mL) were plated in 24-microwell plates and left over night in the CO₂ incubator. Then, cells were treated with 10 μM compounds or with Trolox for 2 h and then continuously stimulated with 1 μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The culture supernatant was collected and mixed in ratio 1:2 with 30% trichloroacetic acid (Kemika, Zagreb, Croatia) and centrifuged 10 min on 3,500 g. After centrifugation supernatant was mixed with TBA (2-thiobarbituric acid, Sigma-Aldrich, St. Louis, MO, USA) solution in a ratio 1:1 and then boiled at 95°C for 30 min. The absorbance was measured at 532 nm using a UV/VIS spectrometer (Lambda 25 UV/VIS, PerkinElmer, USA).
1,1,3,3-Tetraethoxypropane (Sigma-Aldrich, St. Louis, MO, USA) serial dilution standard curve were performed and TBARS values were expressed as μM of malondialdehyde (MDA) equivalents.

**Statistical analysis**

All described experiments were done in triplicate. A nonparametric Mann-Whitney test was applied to analysis antiproliferative effects. Statistical difference between all data groups in an experiment were compared using one-way analysis of variance (ANOVA) pair wise comparison with Dunnett (two sided) analysis. All analyses were done with 95% confidence interval (α=0.05).

**RESULTS AND DISCUSSION**

In this study we examined effects of molecular hybrids with 7-chloroquinoline and arylamidine moieties joined through flexible -NH-CH₂-CH₂-O- linker on induction of oxidative stress and inflammation in the lipopolysaccharides (LPS) stimulated RAW 264.7 cells. To determine optimal concentrations of hybrid compounds for further experiments, antiproliferative effects of 1a–1g were assessed in the non-stimulated RAW 264.7 cells after 72 h of treatment.

As shown in the Figure 2, hybrid compounds 1a–1e in 0.1 to 10 μM range of concentration were not cytotoxic. Only 10 μM compound 1g significantly influenced the growth of the RAW 264.7 cells. Statistically significant decreases in cell survival were observed when hybrid compounds 1a–1g were applied at highest tested concentration of 100 μM. The calculated IC₅₀ values (μM) were for 1a: 49.6±7.2; for 1b: 39.1±6.6; for 1c: 57.5±2.5; for 1d: 45.4±8.0 for 1e: 34.3±1.9; for 1f: 75.4±2.3 and for 1g: 1.6±0.8.

Furthermore, the effects of 10 μM hybrid compounds 1a–1g and Trolox, as a reference compound, on LPS-stimulated RAW 264.7 cells’ growth were also tested. As shown in Figure 3, lipopolysaccharides from *E. coli* (LPS) applied in concentration of 1μg/mL during 24 h had no cytotoxic effect on the growth of RAW 264.7 cells. After 24 h of incubation, Trolox and hybrid compounds 1b, 1d, 1e, and 1f did not show significant effects on the LPS-stimulated cells’ growth. Weak antiproliferative effects were shown by the 1a and 1c, while 1g completely inhibited the LPS-stimulated cells (Figure 3). Based on described results, we excluded the molecular hybrid 1g and selected the 10 μM concentration of hybrid compounds 1a–1f as the optimal concentration for the subsequent experiments.

It is well known that normal cellular metabolic process involving mitochondrial respiratory chain produces ROS while excessive ROS production results in mitochondrial dysfunction which is strongly associated with other pathological conditions, including inflammation (17). Macrophages in vivo and in vitro upon stimulation by LPS exhibit inflammatory responses by excessive accumulation of nitric oxide and superoxide anion whose interaction result in different pathophysiological conditions (11). By analyzing antioxidant capacity of the new synthesized hybrids 1a–1f we wanted to determine if there is a correlation between radical scavenging capacity of tested compounds, redox status in the LPS-stimulated RAW 264.7 cells and their responsiveness to tested compounds.

In our experimental conditions, 1a–1f did not induce changes in ROS levels in the non-stimulated RAW 264.7 cells (data not shown). As shown in the Figure 4, intracellular levels of ROS in the RAW cells stimulated by LPS for 24 h increased almost two times compared
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Since free-radical scavenging is one of the well-known mechanisms by which many compounds act as antioxidants we evaluated in vitro antioxidant capacity of tested compounds by 2,2’-azino-bis(3-ethylbenzthiazoline-

to non-stimulated (control) cells. In LPS-activated RAW 264.7 cells we recorded a slight decrease in the ROS accumulation after treatment with all tested compounds (Figure 4).

Figure 3. Cytotoxic effects of 10 μM quinoline-arylamidine hybrids 1a–1g and Trolox on the RAW 264.7 cells’ growth after pre-treatment for 2 h and stimulation with 1μg/mL LPS for 24 h. LPS: control cells stimulated by lipopolysaccharides (LPS) for 24 h. Cytotoxicity was analysed using the MTT survival assay. The percentage of treated cells growth inhibition was calculated relative to the growth of untreated (control) cells. Data are presented as the mean value ± SD of three independent experiments done in triplicates.

Figure 4. Effect of hybrid compounds 1a–1f on the RAW264.7 intracellular ROS accumulation. Cells (5×10^5 per well in a 96-well plate) were pre-incubated with 10 μM hybrids for 2 h and then continuously stimulated with 1 μg/mL LPS for 24 h. Control: non-treated and non-stimulated cells. LPS: cells stimulated by LPS for 24 h. Data are given as mean fluorescence of ROS level with ± SD. One-way ANOVA with Dunnett analysis (α<0.05) were used to examined differences between control cells and by hybrids treated cells.

Table 1. Radical scavenging capacity (RSC) of tested hybrid compounds 1a–1f

<table>
<thead>
<tr>
<th>Compound</th>
<th>1a</th>
<th>1b</th>
<th>1c</th>
<th>1d</th>
<th>1e</th>
<th>1f</th>
<th>Trolox</th>
<th>Esculetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSC (%)</td>
<td>14.0±2.2</td>
<td>0.2±0.1</td>
<td>3.7±0.3</td>
<td>0.2±0.1</td>
<td>0.6±0.1</td>
<td>1.4±0.2</td>
<td>91.5±2.0</td>
<td>91.4±1.5</td>
</tr>
</tbody>
</table>

*2,2’-Azinobis-3-ethylbenzthiazoline-6-sulfonic acid cation (ABTS') scavenging effect expressed as mean (n=3) values ±SD after 60 minutes of incubation.
6-sulfonic acid (ABTS) radical cation method. Obtained results showed that in vitro scavenger activity of tested hybrids 1a–1f is negligible (0.2 to 14.2 respectively) compared to reference compounds, Trolox and Esculetin (Table 1).

As nitric oxide (NO) is an important pro-inflammatory mediator produced by inducible nitric oxide synthase in macrophages (5, 12), we examined whether or not tested hybrid compounds 1a–1f could reduce the LPS-induced generation of NO in the RAW 264.7 cells. Obtained results showed that LPS stimulation led to statistically significant increase in NO production (to 169.2±9.4 μM) in LPS stimulated cells compared to the non-treated and non-stimulated control cells (26.7±2.4 μM). We found that 10 μM hybrid compounds 1a, 1b, and 1e significantly reduced production of NO in LPS-activated macrophages by about 20–25% compared to LPS induced control cells, indicating their potential as a NO-scavenging compounds. In the applied concentration Trolox had no effect on the NO production in LPS stimulated cells compared to LPS control cells (Figure 5a).

Nitrosative and/or oxidative stress-induced lipid peroxidation is detrimental because it alters the biological properties of the cellular membrane, such as degree of fluidity and inactivated membrane-bound receptors and enzymes, which impairs normal cellular function and increases tissue permeability (18). Therefore, lipid peroxidation products such as malonylaldehyde (MDA) are often used as markers of oxidative/nitrosative damage (19). Thiobarbituric acid (TBA) assay is the most commonly used method for determination of the MDA in biological fluids. As shown in Figure 5b, LPS stimulation of the RAW 264.7 cells increased TBARS production for more than double in the LPS stimulated cells compared to the non-stimulated control cells. The 2 h pretreatment with 10 μM hybrid compounds 1a–1d caused statistically significant decrease in the MDA accumulation in the LPS-stimulated RAW 264.7 cells compared to control cells and completely restored the TBARS content to con-

![Figure 5](image-url)
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CONCLUSIONS

The results presented in this study demonstrate that molecular hybrids with 7-chloroquinoline and arylamidine moieties joined through flexible 2-aminoethanol linker slightly modulated the production of NO and ROS and did not present significant ABTS radical-scavenger activity. At the same time, our results suggest a pharmacological potential of this class of compounds since a markedly decreased accumulation of lipid peroxidation products in the LPS-stimulated RAW 264.7 cells was noticed. Further studies are necessary to investigate mechanisms of action of these compounds. Compounds from this series will serve as a template for the future design of new analogues with improved antimicrobial and anti-inflammatory activities.

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Declaration: The authors declare no competing interests.

REFERENCES