Azulenquinone derivatives have been reported to display a broad spectrum of biological activities, but study at the cellular level has been limited. The effect of twenty-seven azulenquinone derivatives on nitric oxide (NO) production by mouse macrophage-like cells Raw 264.7 was investigated in this study. All of these compounds failed to stimulate the Raw 264.7 cells to produce detectable amounts of NO, but did inhibit NO production by lipopolysaccharide (LPS)-activated Raw 264.7 cells to varying extents. Compounds 7, 8, 9, 13, 16, 25, 27, which showed lesser cytotoxic activity (CC50=425, 381, 482, 179, 119, 235, 225 µM, respectively), inhibited NO production to the greatest extent [selectivity index (SI)= 15.4, 26.2, 3.9, 21.6, 3.1, 6.0, 8.4, respectively]. Western blot and RT-PCR analyses demonstrated that the most active derivatives, 3-morpholino-1,5-azulenquinone [8] and 3,7-dibromo-1,5-azulenquinone [13], significantly reduced both the intracellular concentration of iNOS protein and the expression of iNOS mRNA. ESR spectroscopy showed that compounds [8, 13] weakly scavenged NO produced by NOC-7, possibly via their general reducing activity. These data suggest that the inhibitory effect of NO production by compounds [8, 13] might be generated mostly via the inhibition of iNOS expression, rather than the radical-mediated mechanism.

Azulene derivatives (1-4) have shown several biological activities, including antibacterial activities (5), anti-ulcer activity (6), relaxant activity (7), inhibition of thromboxane A2-induced vasoconstriction and thrombosis (8), acute toxicity and local anesthetic activity (9), and possible chemotherapeutic activity for mucous membrane diseases (10, 11). We have recently reported that methyl 7-isopropyl-2-methoxylazulene-1-carboxylate (12) and 5-aminotropolone (13) induced tumor-specific cytotoxicity and apoptotic cell death (characterized by internucleosomal DNA fragmentation and caspase 3 activation) in HL-60 cells, without (12) or with (13) involvement of the radical-mediated oxidation mechanism, and that 1,3-difluoroazulene (14) and 2,4-dibromo-7-methoxylpropene (15) inhibited nitric oxide (NO) production by LPS-activated mouse macrophage-like Raw 264.7 cells, via partial inhibition of inducible NO synthase (iNOS) expression without involvement of NO radical scavenging activity.

Azulenquinone is a nonbenzenoid aromatic quinone (16, 17) and is also an isomer of naphtoquinone. Naphthoquinones have shown antifungal, antibiotic, antimarial and antitumor activity (18). However, the effects of azulenquinone derivatives on cellular function have not been investigated in detail. We recently found that 3-(3-guaiazulenyl)-1,5-azulenquinone [12] and 7-isopropyl-3-(4-methylazulil)-2-methyl-1,5-azulenquinone [24] (Figure 1) showed relatively higher tumor-specific cytotoxicity and induced apoptosis in human promyelocytic leukemia HL-60 and oral squamous cell carcinoma HSC-2 cells, possibly via the activation of both mitochondria-independent (extrinsic) and -dependent (intrinsic) pathways (19). We investigated here whether twenty-seven azulenquinone derivatives (Figure 1), such as azulenes (14) and tropolons (15), inhibit the nitric oxide (NO) production by unstimulated- and lipopolysaccharide (LPS)-stimulated mouse macrophage-like Raw 264.7 cells. The NO concentration is determined by the intracellular concentration and activity of iNOS protein, and quenching of NO radical, which interacts with superoxide anion (O2-). Therefore, we also investigated whether azulenquinone derivatives affect the expression of iNOS protein and mRNA.
(by Western blot analysis and RT-PCR analyses) and scavenge various radicals, such as NO [generated from 1-hydroxy-2-oxo-3-(N-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7, a NO generator), in the presence of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO, a spin trap agent)] and superoxide anion (O₂⁻) [generated by hypoxanthine (HX) - xanthine oxidase (XOD) reaction in the presence of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO)], by ESR spectroscopy.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco’s modified Eagle medium (DMEM), phenol red-free DMEM (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); HX, XOD, diethylenetriaminepentaacetic acid (DETAPAC), phenylmethylsulfonyl fluoride (PMSF) (Sigma Chem. Co., St. Louis, MO, USA); DMPO, carboxy-PTIO, NOC-7, superoxide dismutase (SOD) from bovine erythrocytes (Dojin, Kumamoto, Japan).


Cell culture. Mouse macrophage-like Raw 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO₂ atmosphere (14, 15).

Assay for cytotoxic activity. The cytotoxic activity of azulenes was determined by the MTT method, and expressed as absorbance at 540 nm of the MTT-stained cells. The 50% cytotoxic concentration (CC₅₀) was determined from the dose-response curve (14, 15).
Assay for NO concentration. Near-confluent Raw 264.7 cells were incubated for 24 h with each test sample in phenol red-free DMEM supplemented with 10% FBS, and the NO production by Raw 264.7 cells was quantified by Greiss reagent, using the standard curve of NO₂⁻. To eliminate the interaction between the sample and Greiss reagent, the NO concentration was measured in the culture medium without the cells, and this value was subtracted from that obtained with the cells. The concentration that inhibited the LPS-stimulated NO production by 50% (50% inhibitory concentration: IC₅₀) was determined from the dose-response curve (13, 14). The efficacy of inhibition of NO production was estimated by the selectivity index SI, which was calculated by the following equation:

\[ SI = \frac{CC₅₀}{IC₅₀} \]

Western blotting. The cell pellets were lysed with 100 μL of lysis buffer (10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF) for 10 min in ice water, and then incubated for 50 min at 4°C with RT-5 ROTATOR (Titec, Saitama, Japan). The cell lysates were centrifuged at 16,000 × g for 20 min at 4°C to remove the insoluble materials and the supernatant was collected. The protein concentrations of the supernatant were measured by Protein Assay Kit (Bio Rad, Hercules, CA, USA). An equal amount of the protein from cell lysates (10 μg) was mixed with 2 x sodium dodecyl sulfate (SDS)-sample buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromophenol blue, 1.2% 2-mercaptoethanol), boiled for 10 min, applied to the SDS-7% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in phosphate-buffered saline [PBS(-)] plus 0.05% Tween 20 for 90 min and incubated with anti-iNOS antibody (1:1,000, Santa Cruz Biotechnology, Delaware, CA, USA) for 90 min at room temperature or overnight at 4°C, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. Immunoblots were detected by Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA).

Assay for mRNA expression. Total RNA was isolated by the PURESCRIPT RNA Isolation kit (Gentra systems) protocol. Raw 264.7 cells were lysed in 300 μL cell lysis solution, and then 100 μL Protein-DNA precipitation solution was added. Cell lysates were centrifuged at 15,000 × g for 3 min. To the supernatant, 300 μL isopropanol was added. After centrifugation at 15,000 × g for 3 min, the pellet was washed in 300 μL 75% ethanol. After centrifugation at 15,000 × g for 1 min, the pellet was air dried for 15 min and dissolved in DEPC-treated H₂O. A reverse transcriptase reaction (RT) was performed with 1.0 μg of total RNA, using the Rever Tra Ace (Toyobo Co., Ltd.), using oligo (dT)₂₀ primer (14, 15). Single strand cDNA, obtained by RT reaction, was amplified, using the KOD plus (Toyobo Co., Ltd.), using iNOS specific primers (5'-CCCTTCCGAAAGGTTTGCAGACGCG-3' and 5'-GGCTGTCAGACCCTTGGCTTTGG-3') and β-actin specific primers (5'-GAGGCCGAGAAGCAAGAGGAGG-3', 5'-TACATGGCTGGGGTCTGAA-3'), according to the protocol. The RT-PCR products were applied to 2% agarose gel, and the ethidium bromide-stained gel was then photographed under UV light.

Radical scavenging activity. The radical intensity of azulenequinones was determined at 25°C, using ESR spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency) (19). For determination of O₂⁻ produced by HX and XOD reactions (total volume: 200 μL) [2 mM HX in 0.1 M phosphate buffer (pH 7.4) (PB) 50 μL, 0.5 mM DETA/PAC 20 μL, 8% DMPO 30 μL, sample (in DMSO) 40 μL, H₂O or SOD 30 μL, XOD (0.5 U/mL in PB) 30 μL], the following instrument settings were used: center field, 336.0±5.0 mT; microwave power, 5 mW; modulation amplitude, 0.1 mT; gain, 400; time constant, 0.1 s; scanning time, 2 min (13-15). The O₂⁻ scavenging activity was expressed as SOD units/mg sample, by calibration with the standard curve of erythrocyte SOD.

The radical intensity of NO, produced from the reaction mixture of 20 μM carboxy-PTIO and 80 μM NOC-7, was determined in 0.1 M phosphate buffer, pH 7.4 in the presence of 30% DMSO. The microwave power and gain were changed to 5 mW and 125, respectively. When NOC-7 (NO generator) and carboxy-PTIO (spin trapping agent) were mixed, NO was oxidized to NO₂⁻ and carboxy-PTIO was reduced to carboxy-PTI, which produces seven-line signals. The NO radical intensity was defined
as the ratio of signal intensity of the first peak of carboxy-PTI to that of MnO (14, 15).

Results

Relationship between cytotoxicity and inhibition of LPS-stimulated NO production. LPS (100 ng/mL) treatment enhanced the NO production by Raw 264.7 cells, from the background level (<1 μM) to 20-25 μM (Figure 2). On the other hand, all azulenequinone derivatives alone failed to stimulate the Raw 264.7 cells to produce any detectable amount of NO (data not shown).

We first investigated the cytotoxic activity of twenty-seven azulenequinone derivatives against Raw 264.7 cells, determined without LPS (Table I). Compound [21] showed the highest cytotoxicity (CC50= 6 μM), followed by [1] (CC50= 9 μM) > [5] (CC50=11 μM) > [20] (CC50= 21 μM) > [12] (CC50= 27 μM) > [11] (CC50= 28 μM) > [4] (CC50= 42 μM) > [6] (CC50= 44 μM) > [15, 26] (CC50= 46 μM) > [22] (CC50= 57 μM). These compounds inhibited NO production by LPS-activated Raw 264.7 cells to varying extents (SI=1.8, 2.8, 2.7, 11.4, 12.6, 4.6, 2.7, 3.6, 17.1 and 2.4, respectively) (mean SI value=5.9±5.3).

Compounds [7, 8, 9, 13, 16, 25, 27], which showed lesser cytotoxic activity (CC50>425, 381, 482, 179, 119, 235, 225 μM, respectively), inhibited NO production to the greatest extent (SI= 15.4, 26.2, 3.9, 21.6, 3.1, 6.0 and 8.4, respectively) (mean SI value=12.1±9.1). Compounds [2, 3, 14, 17, 18, 23], which were the least cytotoxic (CC50>500 μM), inhibited NO production to a lower extent (SI=4.6, 8.6, 10.9, 8.0, 16.0, 3.0, 3.9, respectively) (mean SI value=7.9±4.6).

\[ \text{Table I. Inhibition of NO production by LPS-stimulated Raw 264.7 cells by azulenequinones.} \]

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW.</th>
<th>CC(_{50}) (μM)</th>
<th>IC(_{50}) (μM)</th>
<th>SI</th>
</tr>
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<tr>
<td>(+)LPS</td>
<td>(+)LPS</td>
<td>(+)LPS</td>
<td>(+)LPS</td>
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<td>3</td>
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<td>19</td>
<td>7</td>
</tr>
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<td>[27]</td>
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<td>225</td>
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a: not determined.

iNOS expression. Since 3-morpholino-1,5-azulenequinone [8] and 3,7-dibromo-1,5-azulenequinone [13] most effectively inhibited NO production by LPS-activated Raw 264.7 cells (SI=26.2 and 21.6, respectively) (IC50=12 and 7.6 μM,
respectively), we next investigated whether these compounds reduce the intracellular concentration of iNOS, using Western blot analysis. Unstimulated Raw 264.7 cells expressed only background levels of iNOS protein, but, upon stimulation with LPS, the cells began to express detectable amount of iNOS protein. The LPS-induced iNOS expression was dose-dependently inhibited by simultaneous addition of 3-morpholino-1,5-azulenequinone [8] or 3,7-dibromo-1,5-azulenequinone [13] (Figure 3). The IC_{50} for iNOS protein expression after addition of [8] or [13] was 3.1 and 9.3 μM, respectively.

RT-PCR analysis demonstrated that unstimulated Raw 264.7 cells expressed a trace amount of iNOS mRNA, and treatment of Raw 264.7 cells with either 3-morpholino-1,5-azulenequinone [8] or 3,7-dibromo-1,5-azulenequinone [13] dose-dependently reduced this endogenous iNOS mRNA expression (Figure 4). The levels of iNOS mRNA were significantly enhanced by LPS (100 ng/mL), and the LPS-enhanced iNOS mRNA synthesis was also dose-dependently reduced by these compounds [8, 13] (IC_{50}=10.0 and >22.8, respectively) (Figure 4).

Table II. Radical scavenging activity of 3-morpholino-1,5-azulenequinone [8] and 3,7-dibromo-1,5-azulenequinone [13], 3-morpholino-1,7-azulenequinone [18] and 7-isopropyl-3-morpholino-1,5-azulenequinone [26].

<table>
<thead>
<tr>
<th>Compound</th>
<th>Scavenging activity against NO(\text{a)}) (IC_{50}: μg/mL)</th>
<th>(O_2^-) (SOD unit/mg)</th>
</tr>
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<tbody>
<tr>
<td>[8]</td>
<td>554.0</td>
<td>0.8</td>
</tr>
<tr>
<td>[13]</td>
<td>609.0</td>
<td>145.0</td>
</tr>
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<td>[18]</td>
<td>687.0</td>
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</tr>
<tr>
<td>[26]</td>
<td>No activity(\text{b)})</td>
<td>1.9</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2.4</td>
<td>6125.0</td>
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</table>

\(\text{a)}\) determined at 3 min after mixing.

\(\text{b)}\) NO scavenging activity was not detected.

Radical scavenging activity. ESR spectroscopy shows that compounds [8, 13, 18] scavenged NO produced by NOC-7 (IC_{50}=554-687 μg/mL), to an extent much lower than that attained by gallic acid (a positive control) (IC_{50}=2.4 μg/mL). All samples reduced the intensity of carboxy-PTI by 50-80%, suggesting their general reducing activity. Therefore, the exact NO scavenging activity may be slightly higher than that expressed in Table II.

Compound [13] potently scavenged \(O_2^-\) generated by the HX-XOD reaction (145 SOD unit/mg), but to a much lower extent than that attained by gallic acid (6125 SOD unit/mg). On the other hand, [8, 18, 26] (0.8, 2.0 and 1.9 SOD unit/mg, respectively) were much less active (Table II).

Discussion

The present study demonstrated that azulenequinones generally showed higher cytotoxicity and SI values than azulenes (14). The parent compound, 1,5-azulenequinone [1], showed relatively higher cytotoxicity and lower selectivity of the inhibition of LPS-stimulated NO production by Raw 264.7 cells (CC_{50}=9 μM; SI=2.8). Similarly, 3-(4-nitrophenoxy)-1,5-azulenequinone [6] and 3-bromo-2-methoxy-1,5-azulenequinone [21] showed relatively higher cytotoxicity and low selectivity (CC_{50}=44 and 6 μM, respectively; SI=2.7 and 1.8, respectively).

1,5-Azulenequinones showed comparable SI values with the corresponding 1,7-azulenequinones: 3-bromo-1,5-
Azulenequinones with the substituted azulene ring, such as 3-(1-azulenyl)-1,5-azulenequinone [11] and 3-[(1,2-dihydroxyethyl)amino]-7-isopropyl-1,5-azulenequinone [26], showed higher cytotoxicity and SI values (CC_{50}=28 and 27 μM, respectively; SI=12.6 and 11.4, respectively).

In general, amino-derivatives such as 3-[(N,N-dimethyloxy)-1,5-azulenequinone [8], 3-[(N,N-dimethyloxy)-1,7-azulenequinone [17], 3-morpholino-1,5-azulenequinone [8] (SI=26.2) and 3-morpholino-1,7-azulenequinone [18] (SI=16.0); 7-isopropyl-3-[(N,N-dimethyloxy)-1,5-azulenequinone [25] (SI=6.0) and 3-[(N,N-dimethyloxy)-5-isopropyl-1,5-azulenequinone [23] (SI=3.9).

Among hologenated derivatives, 3-bromo-1,5-azulenequinone [3], 3,7-dibromo-1,5,1-azulenequinone [13] and 3-bromo-1,7-azulenequinone [14] showed the relatively higher SI values (SI=8.6, 21.6 and 10.9, respectively).

Addition of an alkyl group consistently reduced the SI value of the parent compound: 3-bromo-1,5-azulenequinone [3] (SI=8.6) and 3-bromo-2-methyl-1,5-azulenequinone [20] (SI=2.5); 3-[(N,N-dimethyloxy)-1,5-azulenequinone [7] (SI=15.4) and 7-isopropyl-3-[(N,N-dimethyloxy)-1,5-azulenequinone [25] (SI=6.0); 3-morpholino-1,5-azulenequinone [8] (SI=26.2) and 7-isopropyl-3-morpholino-1,5-azulenequinone [26] (SI=17.1).

In general, methoxy- and phenoxy-derivatives showed relatively higher cytotoxicity but lower SI values: 3-phenoxo-1,5-azulenequinone [5] (CC_{50}=1.1 μM, SI=2.7); 3-(4-nitrophenoxy)-1,5-azulenequinone [6] (CC_{50}=44 μM, SI=2.7), 3-(4-nitrophenoxy)-1,7-azulenequinone [16] (CC_{50}=119 μM, SI=3.1) and 3-bromo-2-methoxy-1,5-azulenequinone [21] (CC_{50}=6 μM, SI=1.8).

The present study demonstrated that the most active compounds [8, 13] inhibited NO production by activated macrophages mainly via the inhibition of iNOS protein expression. Compound [8] almost completely inhibited iNOS mRNA expression, whereas [13] partially inhibited the iNOS mRNA expression, by an, as yet, unknown mechanism. We found that [13] not only scavenged the O_2^- radical (Table II), but also produced the carbon radical (data not shown), suggesting the bimodal action of this compound, i.e. antioxidant action at lower concentration and pro-oxidant action at higher concentrations. The present study suggests that compounds [8, 13] may modify the function of macrophages. Further studies are required to demonstrate the anti-inflammatory action of these compounds.

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References

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