Anti-inflammatory effects of *Punica granatum* Linne *in vitro* and *in vivo*

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**A R T I C L E   I N F O**

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Inducible nitric oxide synthase
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**A B S T R A C T**

Inflammation can cause various physical dysfunctions. *Punica granatum* Linne (pomegranate), a high phe-
monic content fruit, is widely used as an antipyretic analgesic in Chinese culture. Pomegranate has shown
potential nitric oxide (NO) inhibition in LPS-induced RAW 264.7 macrophage cells. Moreover, pomegran-
ate (100 mg/kg) significantly decreased carrageenan-induced mice paw edema for 1, 3, 4, and 5 h. There-
fore, column chromatography combined with *in vitro* bioassay-guided fractionation was used to isolate
the active anti-inflammatory components from the pomegranate. Punicalagin (1), punicalin (2), strictinin
A (3), and granatin B (4) were obtained with yields of 0.093%, 0.015%, 0.003%, and 0.013%, respectively. All
these hydrolysable tannins inhibited NO production and iNOS expression in RAW 264.7 cells. Among
them, 4 showed the strongest iNOS and COX-2 inhibitory effects, and exhibited these effects in the inhibi-
tion of paw swelling and the PGE2 level in carrageenan-induced mice. Taken together, we suggest that 4
could be used as a standard marker for the anti-inflammatory effect of pomegranate.

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1. Introduction

*Punica granatum* Linne, pomegranate (Punicaceae), a common fruit in the Mediterranean and Iran, is widely used for therapeutic formulae, cosmetics, and food seasoning. Pomegranate, also easily acquired from traditional medicine markets, was usually used as an astringent agent (Alper & Acar, 2004), for eliminating parasites (Mudzhiri, 1954; Raj, 1975) and as an antipyretic. The pharmacological functions of pomegranate include antioxidation (Lansky & Newman, 2007), anti-tumour (Khan, Afaq, Kweon, Kim, & Mukhtar, 2006), anti-hepatotoxicity (Kaur, Jabbar, Newman, 2007), anti-tumour (Khan, Afaq, Kweon, Kim, & Mukhtar, 2006), anti-hepatotoxicity (Kaur, Jabbar, Newman, 2007), anti-lipoperoxidation (Reddy, Gupta, Jacob, Khan, & Ferreira, 2006) and anti-bacteria properties (Menezes, Cordeiro, & Viana, 2006). In hematology, pomegranate could reduce the common carotid intima-medium thickness, thus lowering blood pressure and decreasing low-density lipoprotein (LDL) oxidation and the incidence of heart disease (Aviram et al., 2002, 2004). In our previous studies, we found that extract from the dried peel of the pomegranate could significantly inhibit NO production. Hence, we suggested pomegranate contains the anti-inflammatory activity components.

In the last few years, many important functions of fresh fruits and vegetables have been reported, and they are now recognised as being good sources of natural antioxidants (Joseph, Shukitt-Hale, & Lau, 2007), such as grapes, apples, and guavas. The antioxidants can prevent lipid peroxidation, and DNA and protein damage. Polyphenols have been acknowledged to have health-beneficial effects, owing to derived products such as flavonoids, tannins, coumarins, and lignans. According to recent reports, the pomegranate is rich in polyphenols, including mainly ellagitannins, gallotannins (punicalin, punicalagin, pedunculagin, pungilucin, granatin B, and tellimagrandin I) (Satomi et al., 1993) and anthocya-

dins (delphinidin, cyanidine and pelargonidin) (Noda, Kaneyuki, Mori, & Packer, 2002). However, the correlation between the phytochemicals and the anti-inflammatory properties of the dried peel of the pomegranate has not been investigated. Therefore, this study aimed to clarify the anti-inflammatory activities of pomegranate and its active components.

Inflammation, the first physiological defense system in the human body, can protect against injuries caused by physical wounds, poisons, etc. This defense system, also called short-term inflammation, can destroy infectious microorganisms, eliminate irritants, and maintain normal physiological functions. However, long-term over-inflammation might cause dysfunctions of the regular physiology, i.e., asthma and rheumatic arthritis.

We used *in vitro* and *in vivo* models to confirm the anti-inflammatory activity of pomegranate. Lipopolysaccharide (LPS)-induced RAW 264.7 murine macrophages were used in the *in vitro* study, and carrageenan-induced paw edema in mice served as the *in vivo* study. LPS can induce several cytokines, such as prostaglandins and nitric oxide (NO), which are involved in pro-inflammatory processes. NO can kill bacteria and viruses and is also an important
Fig. 1. Isolation flowchart of *Punica granatum* L. using anti-inflammation bioassay-guided fractionation. Numbers in the parentheses were the IC\textsubscript{50} values (\(\mu\)g/ml) of NO inhibition. All test fractions displayed less than 10% cytotoxicity at 200 \(\mu\)g/ml, except D20M, D40M, D20M – TH\(2\)O, and D20M – T60M. The IC\textsubscript{50} values of the test sample exceeded 200 \(\mu\)g/ml.
mediator of vasodilatation; but too much NO might cause hypotension or septicemia (Moncada & Higgs, 2006). Prostaglandins have many physiological activities, such as inducing inflammation. Many tissues acutely or chronically generate excess NO and prostaglandin E2 (PGE2) by the overexpression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the presence of various inflammatory stimulators. RAW 264.7 cells induced by LPS can produce the overexpression of NO and PGE2, and their regulatory proteins, iNOS and COX-2. Therefore, we used this strategy, combined with chromatography, to isolate the active components, and we used carrageenan-induced paw edema in mice to confirm the in vivo anti-inflammatory effects of pomegranate.

2. Materials and methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yi) 2,5-diphenyltetrazolium bromide (MTT), indomethacin, N-nitro-L-arginine methyl ester (L-NAME), and the other chemicals were purchased from Sigma Industries (St. Louis, MO, USA). Dubesco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotics, and glutamine were purchased from GIBCO BRL (Grand Island, NY, USA). Diaion HP-20 gels were bought from Mitsubishi Chemical Industry (Tokyo, Japan). Western blotting was performed using an antibody specific to mouse. iNOS (sc-650), anti-COX-2 (sc-1745), and anti-GAPDH (sc-32233) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Sample preparation

Test solutions of pomegranate were prepared by dissolving pomegranate in 10% DMSO, which was then stored at 4°C and used within 1 month. Serial dilutions of test solutions with culture medium were prepared before the in vitro assays.

2.3. Determination of total phenols

The total phenol content was determined by the Folin-Ciocalteu method. Pomegranate extract was dissolved in distilled water and mixed with the Folin-Ciocalteu reagent and 7.5% aqueous Na2CO3 solution. After standing for 5 min at 50°C, the absorbance was measured at 600 nm in an ELISA reader.

2.4. Determination of the total flavones content

The total flavonal content was determined by the vanillin assay. Vanillin was dissolved in 80% H2SO4 to prepare the vanillin reagent. Pomegranate extract was dissolved in the distilled water and mixed with the vanillin reagent. After standing for 15 min at room temperature, the absorbance was measured at 530 nm in an ELISA reader. The amount of total flavanol was expressed as catechin equivalents (CE, μg catechin/mg sample) through the calibration curve of catechin. The calibration curve ranged from 7.8 to 250 μg/ml (R² = 1) (He, Liu, & Liu, 2008).

2.5. Determination of NO produced from LPS-induced RAW 264.7 cells

RAW 264.7 cells, a murine macrophage cell line, was obtained from American Type Cell Culture (ATCC no. TIB-71; Rockville, MD, USA). Cells were cultured in DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin, and maintained at 37°C and 5% CO2. RAW 264.7 cells (4.0 × 10⁵ cells/ml) were seeded in 96-well plates and then co-treated with LPS (500 ng/ml) and the test samples. After 18 h, the NO production was determined by mixing the culture supernatant with Griess reagent, and the absorption was detected in an ELISA reader at 530 nm. Anti-inflammatory activity was presented in terms of the NO production inhibition percentage. The viability of RAW 264.7 cells was detected by the MTT assay (Tseng, Lee, Chen, Wu, & Wang, 2006).

2.6. Isolation of polyphenols from P. granatum

Dried pomegranate peels (600 g) were purchased from a traditional Chinese medicine store in Taipei, pulsed and filtered through a 20 mesh. Pulverised peels were then homogenised with 70% aqueous acetone (3 L × 4) and the homogenate was then filtered. The filtrate was concentrated by evaporation under reduced pressure (ca. 40°C) and further freeze-dried to yield the 70% acetone extract (330 g, yield 55%). The 70% acetone extract was dissolved in water and then partitioned with n-hexane to remove the non-polar compounds. The aqueous layer was chromatographed over a Diaion HP-20 gel column (9.5 × 40 cm) with stepwise aqueous MeOH (H2O – 20% MeOH – 40% MeOH – 60% MeOH – 70% acetone). Column chromatography was combined with an NO inhibition assay to clarify the bioactive fractions. We found that the 20% MeOH (D20M) and 40% MeOH (D40M) fractions of the Diaion column exhibited greater NO inhibition. Furthermore, the D20M fraction was chromatographed on a Toyopearl HW-40 (C) (Tosoh Bioscience, Montgomeryville, PA, USA) gel column (2.5 × 40 cm) and eluted stepwise with H2O – 70% MeOH – MeOH–acetone–H2O (8:1:1:1, v/v) → MeOH–acetone–H2O (7:2:1, v/v/v) → MeOH–acetone–H2O (6:3:1, v/v/v) → 70% acetone. The 70% MeOH (D20M – T70M) eluate was applied to an ODS column (0.05% trifluoroacetic acid:CH3CN, 92:8) and HLB extraction cartridges (Waters, Milford, MA, USA) to yield punicalin (556 mg, 1) and punicanil (87 mg, 2). The D40M fraction was chromatographed on a Toyopearl HW-40 (C) gel column (2.5 × 40 cm) and eluted stepwise with H2O – 70% MeOH – MeOH–acetone–H2O (8:1:1:1, v/v) → MeOH–acetone–H2O (7:2:1, v/v/v) → MeOH–acetone–H2O (6:3:1, v/v/v) → 70% acetone. The 60% MeOH eluate (D40M – T60M) was purified on an ODS column with 0.05% trifluoroacetic acid–CH3CN (88:12) to obtain 15 mg of strictinin A (3). The

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>LPS-induced RAW 264.7 macrophage cells</th>
<th>Free radical scavenging activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO inhibitiona</td>
<td>iNOS activityb,c</td>
</tr>
<tr>
<td></td>
<td>IC50 values (nM)</td>
<td>(%)</td>
</tr>
<tr>
<td>1</td>
<td>69.8</td>
<td>15.7 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>78.6</td>
<td>46.7 ± 7.0</td>
</tr>
<tr>
<td>3</td>
<td>63.1</td>
<td>25.4 ± 2.4</td>
</tr>
<tr>
<td>4</td>
<td>33.6</td>
<td>14.8 ± 1.0</td>
</tr>
<tr>
<td>l-NAMEde</td>
<td>–</td>
<td>46.4 ± 2.9</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SD of three experiments.

a Co-treatment of LPS and each test compound for 18 h and no cytotoxicities were found in these IC50 values.
b RAW 264.7 cells were stimulated with 1 μg/ml LPS and incubated overnight.
c Each test compound was 100 μM.
d l-NAME was used as positive control and its concentration was 400 μM.
70% MeOH eluate (D40M – T70M) was purified on an ODS column with 0.05% trifluoroacetic acid–CH₃CN (85:15) and 0.05% trifluoroacetic acid–EtOH–EtOAc (100:10:5, v/v/v) to obtain 78 mg of granatin B (4) (Fig. 1). The structures of these four compounds were identified by nuclear magnetic resonance and the purity of each compound (>99.0%) was confirmed by high-performance liquid chromatography. The formulae and molecular weights of punicalagin (1), punicalin (2), strictinin A (3), and granatin B (4) are C₄₃H₂₈O₃₀ (MW 1048.7), C₃₄H₂₂O₂₂ (MW 782.53), C₄₃H₂₈O₃₀ (MW 634.46), and C₄₁H₂₈O₂₇ (MW 952.66), respectively.

2.7. Determination of NO radical-scavenging activity

Sodium nitroprusside (SNP) is a stable NO donor. We used SNP as an NO donor to evaluate the direct NO radical clearance of samples. The SNP solution was prepared with H₂O. Test solution was added to the sample volume of SNP solution (50 mM). The mixture was incubated at 37°C for 5 h. Then, we took the supernatant (1, mixed it with the Griess reagent, and read the absorption on an ELISA reader at 530 nm (Bor, Chen, & Yen, 2006).

2.8. iNOS activity assay in LPS-induced RAW 264.7 cells

Initially, RAW 264.7 cells were pre-stimulated by 1 µg/ml LPS for 24 h to activate the iNOS. Then, cells were collected and washed twice with PBS to remove the excessive LPS, and the activated RAW 264.7 cells (4.0 × 10⁵ cell/ml) were seeded in 96-well plates. Crude extract and active components were immediately added to the cells. The 96-well plate was incubated at 37°C overnight. The NO detecting method was the same as previously described. We used N-nitrol-arginine methyl ester (L-NAME, 400 µM), a specific inhibitor of NO synthase enzyme, and as the positive control.

2.9. iNOS and COX-2 expression assay in LPS-induced RAW 264.7 cells

Total cellular protein was extracted with a RIPA solution (radioimmuno-precipitation assay buffer) at −20°C overnight. We used BSA (bovine serum albumin) as a protein standard to calculate equal total cellular protein amounts. Protein samples (15 µg) were resolved by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using standard methods, and then were transferred to nitrocellulose (Hybond-PVDF) membranes by electroblotting and blocking with 1% BSA. The membranes were probed with the primary antibodies (iNOS, COX-2 and GAPDH) at 4°C overnight, washed three times with PBST, and incubated for 1 h at 37°C with alkaline phosphatase-conjugated secondary antibodies. Then, we used an NBT/BCIP commercial kit (Gibco) as the visualising agent. The intensity of the bands was quantified by computer-assisted image analysis using Alphalnnotech Digital Imaging Systems. Western blotting results are representative of three independent experiments for every data point.

2.10. Model of 1% carrageenan-induced paw edema in ICR mice

Male ICR mice weighing 25 ± 2 g were bought from the National Science Council, Taipei, Taiwan, and maintained at 21 ± 2°C with food and water ad libitum. They were kept on a 12-h light/12-h dark cycle. All mice used in this experiment were cared for according to
the Ethical Regulations on Animal Research of our university. Edema in the left hind paw of the mice was induced by injecting 50 µl of 1% (w/v) carrageenan into the subplantar region. The perimeter of the paw was measured 1 h before the carrageenan injection and after 1–6 h, using calipers. One hour before the injection, the pomegranate (100 mg/kg), the active component (2.5 and 10 mg/kg), and indomethacin (10 mg/kg, as a positive control) were given orally, while the control group was given distilled water. The blank group was injected with normal saline and given distilled water. Each group consisted of five animals. After 6 h, mice were sacrificed and the serum collected for the PGE2 measurement (Tseng et al., 2006).

2.11. Measurement of PGE2 production

Serum and cell culture mediums were determined the PGE2 concentrations by the PGE2 ELISA kit (Amersham Pharmacia Bio-tech, Buckinghamshire, UK).

2.12. Statistical analysis

The data are presented as mean and standard deviation (SD). Significance was calculated using the Student’s t-test. Differences were considered significant for $p < 0.05$.

### Table 1: PGE2 production

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Fold 1.00</th>
<th>1.25</th>
<th>2.50</th>
<th>5.00</th>
<th>10.00</th>
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<tbody>
<tr>
<td>(A) LPS (8h)</td>
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<td>1.47</td>
<td>1.60</td>
<td>1.94</td>
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<td>(B) LPS (8h)</td>
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<td>1.30</td>
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<tr>
<td>(D) LPS (8h)</td>
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### Table 2: PGE2 production

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<td>1.15</td>
<td>1.42</td>
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3. Results

3.1. Polyphenols isolation from P. granatum L.

The phytochemical components of the 70% acetone extract of pomegranate peels were screened using the Folin-Ciocalteu method and vanillin assay. The screening found that pomegranate was rich in total phenol and flavanol (471.0 ± 32.0 µg gallic acid equivalent/mg in total phenol and 257.0 ± 19.6 µg catechin equivalent/mg in total flavanol).

The NO inhibition bioassay-guided fractionation flowchart of pomegranate is shown in Fig. 1. These four hydrolysable tannins, punicalagin (1), punicalin (2), strictinin A (3), and granatin B (4), were isolated from D20M and D40M of the pomegranate fractions, with stronger inhibition of NO production; the yields were 0.093%, 0.015%, 0.003% and 0.01%, respectively.

3.2. NO inhibitory effects of four hydrolysable tannins

The NO inhibitory effects of 1–4 were measured in LPS-induced RAW 264.7 cells for 18 h. We collected the culture medium to detect the NO levels using the Griess reaction. Compounds 1–4 showed less than 10% cytotoxicities in LPS-induced RAW 264.7 cells for 18 h. Compound 4 displayed a more potent NO inhibition effect.
iNOS protein expression in LPS-induced RAW 264.7 cells for 8 and 18 h were observed by Western blot assay. As shown in Fig. 3, the four hydrolysable tannins displayed dose-dependently inhibitory effects on the iNOS expression at 12.5–100 μM. In addition, the 8 h group expressed more significantly iNOS inhibitory effects than the 18 h group. Therefore, these four hydrolysable tannins could decrease the iNOS protein expressions from the early stage (8 h) to the late stage (18 h). Taken together, 1–4 significantly decreased the iNOS expressions in LPS-induced RAW 264.7 cells, and 4 was the strongest among them.

On the other hand, RAW 264.7 cells were pretreated with LPS for 24 h to observe the iNOS activity inhibition of these four hydrolysable tannins. As seen in Table 1, L-NAME acted as a positive control and displayed significant iNOS activity inhibition at 400 μM. However, these four hydrolysable tannins had no iNOS activity inhibitory effects and even exhibited cytotoxicity at 100 μM. Hence, these four hydrolysable tannins inhibited NO production through directly decreasing the iNOS protein expressions.

3.4. COX2 and PGE2 inhibitory effects of four hydrolysable tannins

The COX-2 and PGE2 inhibitory effects of these four hydrolysable tannins were evaluated in LPS-induced RAW 264.7 cells for 8 and 18 h. As shown in Fig. 3, Compound 4 inhibited COX-2 protein expression in dose-dependent (Fig. 3) and PGE2 productions (Fig. 4A) more significantly than the others after treatment with LPS for 8 h. Moreover, 4 inhibited PGE2 productions in a dose-dependent manner, and the IC50 value was 66.22 ± 9.4 μM (Fig. 4D). However, COX-2 protein expression of LPS-induced RAW 264.7 cells was not inhibited after treatment with the four hydrolysable tannins for 18 h (Fig. 3). Hence, we suggested that 4 could inhibit COX-2 and PGE2 production in the early stage (8 h) because there was less COX-2 expression at that stage, but in the late stage (18 h), COX-2 expression reached a stable state, so 4 had difficulty inhibiting its expression.

3.5. Inhibitory effect of pomegranate and 4 on carrageenan-induced paw edema in ICR mice

Carrageenan-induced paw edema in ICR mice was used to evaluate the in vivo anti-inflammatory model. We used indomethacin, a common non-steroidal anti-inflammatory drug (NSAID) as a positive control. Mice were treated with pomegranate (100 mg/kg) or indomethacin (10 mg/kg) 1 h before carrageenan induction, and had detected paw edema for 6 h. Results showed that carrageenan significantly induced paw edema during the entire experiment in the control group (Table 2). Pomegranate significantly reduced paw edema by more than 50% at 1–5 h after the carrageenan injection, and had even greater potential paw edema inhibitory effects than indomethacin.

Table 2: Effects of pomegranate and granatin B on the paw perimeter of carrageenan-induced mice paw edema.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time after injection of carrageenan (h)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1.74 ± 0.30</td>
<td>1.41 ± 0.17</td>
<td>1.41 ± 0.23</td>
<td>1.21 ± 0.13</td>
<td>0.92 ± 0.16</td>
<td>0.88 ± 0.08</td>
</tr>
<tr>
<td>Indomethacin (10 mg/kg)</td>
<td></td>
<td>0.70 ± 0.15</td>
<td>0.90 ± 0.34</td>
<td>0.79 ± 0.33</td>
<td>0.72 ± 0.15</td>
<td>0.75 ± 0.09</td>
<td>0.62 ± 0.19</td>
</tr>
<tr>
<td>Pomegranate (100 mg/kg)</td>
<td></td>
<td>0.86 ± 0.10</td>
<td>0.60 ± 0.07</td>
<td>0.59 ± 0.28</td>
<td>0.42 ± 0.30</td>
<td>0.4 ± 0.36</td>
<td>0.34 ± 0.50</td>
</tr>
<tr>
<td>Granatin B (4)</td>
<td></td>
<td>1.68 ± 0.13</td>
<td>0.84 ± 0.20</td>
<td>0.77 ± 0.07</td>
<td>0.75 ± 0.17</td>
<td>0.63 ± 0.03</td>
<td>0.59 ± 0.15</td>
</tr>
</tbody>
</table>

Values were presented as the mean ± SD of the increasing mice paw edema (mm) of four animals for each group. ∗ p < 0.05. ∗∗ p < 0.001 were presented as significantly different from the control.
Based on the structural similarities of these four hydrolysable tannins, such as punicalagin, punicalin, pedunculagin, and granatin B, were isolated from pomegranate by bioassay-guided fractionation. Each of them displayed a dose-dependently and significantly inhibitory effect on NO production in LPS-induced RAW264.7 cells (Table 1). Furthermore, granatin B (4) more strongly inhibited PGE2 production and COX-2 expression in LPS-induced RAW264.7, and that COX-2 expression was significantly inhibited at 8 h and reached a stable state for 18 h. Unlike NO production, activated COX-2 could convert arachidonic acid to PGE2 in only 30 min. Hence, the COX-2 and PGE2 inhibitory effects of 4 from LPS-induced RAW 264.7 at 8 h were stronger than those at 18 h. In summary, we suggest that 4 is an effective anti-inflammatory compound and has dual roles in anti-inflammation, by decreasing PGE2 production in the early stage and decreasing NO production in the late stage.

In conclusion, 4 not only displayed the best NO inhibitory abilities in LPS-induced RAW 264.7, but also had the strongest PGE2 inhibitory effects in the in vitro assay. Taken together, 4 could be used as a standard marker compound to determine the potential anti-inflammatory effect of pomegranate.

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