Modulatory Effect of Bolinaquinone, a Marine Sesquiterpenoid, on Acute and Chronic Inflammatory Processes

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ABSTRACT

The marine metabolite bolinaquinone is a novel inhibitor of secretory phospholipase A2 (sPLA2), with a potency on the human synovial enzyme (group II) higher than that of manno-lide. This activity on the sPLA2 was confirmed in vivo in the 8-h zymosan rat air pouch on the secretory enzyme accumulation in the pouch exudate. Additionally, bolinaquinone decreased potently the synovias and release of leukotriene B4 (LTB4) in calcimagim (A23187)-stimulated human neutrophils as a consequence of the inhibition of 5-lipoxygenase activity, as well as PGE2 and NO production on zymosan-stimulated mouse peritoneal macrophages. This compound exerted anti-inflammatory effects by topical and oral routes on the mouse ear edema induced by 12-O-tetradecanoylphorbolacetate, with ID50 values of 76.7 μg/ear and 5.6 mg/kg, respectively, with a significant decrease in PGE2, LTB4, and tumor necrosis factor-α (TNF-α) levels being more effective than indomethacin. This effect was confirmed in the mouse paw carrageenan edema after oral administration. Moreover, bolinaquinone was able to reduce the inflammatory response of adjuvant arthritis by inhibiting PGE2, NO, and TNF-α production in paw homogenates without affecting PGE2 levels in the stomach. Additionally, bolinaquinone inhibited inducible nitric oxide synthase expression and reduced the degree of bone resorption, soft tissue swelling, and osteophyte formation.

Phospholipases A2 (PLA2) are a class of lipolytic enzymes that release arachidonic acid from the sn-2 position of phospholipids as a previous step for the synthesis of a great variety of eicosanoids and platelet-activating factor (Balsinde et al., 1999). Mammalian PLA2s are divided in two major classes according to their molecular mass and location: intracellular PLA2 and secreted (sPLA2). Type IIA sPLA2 (sPLA2-IIA), the best studied enzyme of sPLA2, has been reported to release arachidonic acid in some systems and may provide the substrate for both cyclooxygenase (COX) and 5-lypoxigenase (5-LO) product formation in mouse bone marrow-derived mast cells (Fonteh et al., 1994). Marine organisms are a rich source of molecules exhibiting PLA2 inhibitory properties in vitro, mainly on secretory enzymes (Garcia-Pastor et al., 1999a,b). In a previous study (Ferrándiz et al., 1994), we reported that avarol, a sesquiterpenoid hydroquinone with weak inhibitory properties against human synovial PLA2, and its quinone derivative, avarone, potently inhibited paw edema induced by carrageenan, as well as ear edema induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). Bolinaquinone, a sesquiterpenoid derivative sharing both hydroquinone and quinone moieties in its chemical structure (Fig. 1), was recently isolated from a Dysidea sp. sponge (Giannini et al., 2001), showing a char-
characteristic in vitro inhibitory profile against different sPLA₂ belonging to the group I (porcine pancreatic enzyme), group IIa (human synovial), and group III (bee venom enzyme) without any effect on cytosolic PLA₂. Its potency was higher than that of manoalide, one of the best-known PLA₂ inhibitors (Soriente et al., 1999; Giannini et al., 2001).

It is known that prostaglandins and nitric oxide (NO) are ubiquitous mediator systems exerting numerous vascular and inflammatory effects (Kaur and Halliwell, 1994; Kang et al., 2001). The peroxidase-conjugated goat anti-rabbit IgG was purchased from DAKO (Copenhagen, Denmark). Chemical Co. (Ann Arbor, MI). The peroxidase-conjugated goat anti-synthase (iNOS) polyclonal antisera were purchased from Cayman Amersham Iberica (Madrid, Spain). COX-2 and inducible nitric oxide synthase (iNOS) polyclonal antisera were purchased from Cayman Amersham Iberica (Madrid, Spain).

The purpose of the present study was to investigate the modulatory effect of this sponge metabolite on acute and chronic inflammatory models, as well as to define its mechanism of action on the basis of its potential modulation of cytokine production, generation of nitric oxide, and eicosanoid metabolites, including prostaglandins and leukotrienes. This is the first report concerning the pharmacological properties of this compound.

Materials and Methods

Reagents. Bolinaquinone was isolated from the sponge Dysidea sp. following known procedures recently published (Giannini et al., 2001). [5,6,8,11,12,14,15(n)-1H]PGE₂, [5,6,8,9,11,12,14,15(n)-1H]LTB₄, [9,10-1H]oleic acid, and the enhanced chemiluminescence system were from Amersham Iberica (Madrid, Spain). COX-2 and inducible nitric oxide synthase (iNOS) polyclonal antisera were purchased from Cayman Chemical Co. (Ann Arbor, MI). The peroxidase-conjugated goat antirabbit IgG was purchased from DAKO (Copenhagen, Denmark). Mycobacterium butyricum was obtained from Difco (Detroit, MI). The remainder of reagents were from Sigma-Aldrich (St. Louis, MO).

Preparation of Human Neutrophils. Leukocytes were obtained and purified as previously described (Bustos et al., 1995). Viability was greater than 95% by the trypsin blue exclusion test. The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Gross and Levi, 1992) was used to assess the possible cytotoxic effect of bolinaquinone on human neutrophils.

Synthesis and Release of LTB₄ by Human Neutrophils. A suspension of human neutrophils (5 × 10⁶ cells/ml) was preincubated with test compound or vehicle and then stimulated with the calcium ionophore A23187 (1 μM) for 10 min at 37°C. LTB₄ levels in supernatants were measured by radioimmunoassay (Hoult et al., 1994). High speed (100,000 g) supernatants from sonicated human neutrophils were obtained and incubated in appropriate conditions with 10 μM arachidonic acid to assess 5-lipoxygenase activity (Tateson et al., 1988).

Isolation and Culture of Mouse Peritoneal Macrophages. Female Swiss mice weighing 25 to 30 g were used to obtain highly purified peritoneal macrophages. Cells were harvested by peritoneal lavage 4 days after i.p. injection of 1 ml of 10% thioglycollate broth. Cells were resuspended in culture medium (120 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl₂ × 2H₂O, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM HEPES, 1 mM arginine, and 10 mM glucose) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and incubated at 37°C for 2 h. The adherent cells were used to perform the following experiments. Cytotoxicity was assessed by the reduction of MTT as above.

iNOS and COX-2 Activity in Mouse Peritoneal Macrophages. Peritoneal macrophages (4 × 10⁶ cells/well) were preincubated with test compounds or vehicle for 30 min and then stimulated with zymosan (0.1 mg/ml) in 96-well culture plate at 37°C for 18 h in the presence of test compounds or vehicle. The nitrite concentration in culture supernatants, as a reflection of NO release, was determined fluorometrically (Misko et al., 1993). PGE₂ levels were determined by radioimmunoassay (Hoult et al., 1994).

Mouse Ear Edema. All studies were performed in accordance with the Declaration of Helsinki and the European Community guidelines for the handling and use of laboratory animals. The protocols were approved by the institutional Animal Care and Use Committee. TPA (2.5 μg/ml) dissolved in 20 μl of acetone was applied in 10-μl volumes to both inner and outer surfaces of the right ear of Swiss mice (20–25 g). Test compounds were applied topically in acetone 30 min before TPA administration or orally in olive oil 1 h before TPA. The animals were killed by cervical dislocation after 4 h, and equal sections of both ears were punched out and weighed. The increase in the weight of the right ear punch over the left indicated the edema (Carlson et al., 1985). The ear sections were homogenized and assayed for wet weight. The ear edema was calculated as percent inhibition calculated as: 100 × [(A - B)/A], where A = weight of ear punch of test and B = weight of ear punch of control.

Table 1

<table>
<thead>
<tr>
<th>18-h Treatment (Induction Phase)</th>
<th align="right">Nitrite</th>
<th align="right">PGE₂</th>
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<tbody>
<tr>
<td>Nonstimulated cells</td>
<td align="right">65.5 ± 6.8**</td>
<td align="right">1.2 ± 0.2**</td>
</tr>
<tr>
<td>Zymosan-stimulated cells</td>
<td align="right">416.8 ± 35.2</td>
<td align="right">7.1 ± 0.6</td>
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<td>% I</td>
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<tr>
<td>1 μM</td>
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<tr>
<td>Bolinaquinone</td>
<td align="right">47.8 ± 4.8**</td>
<td align="right">48.2 ± 3.6**</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td align="right">99.8 ± 1.0**</td>
<td align="right">97.4 ± 4.5**</td>
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**p < 0.01 compared with zymosan-stimulated cells.
in 750 µl of saline, and after centrifugation at 1200 g for 15 min at 4°C, the PGE₂ and LTB₄ content was determined as above. TNF-α levels were quantified by time-resolved fluorimunoassay (Pennon et al., 1995).

**Carrageenan Paw Edema.** The anti-inflammatory activity of bolinaquinone was assessed by the carrageenan paw edema test in mice according to the method of Sugishita et al. (1981). This compound (3.1, 6.3, 12.5, and 25 mg/kg), indomethacin (5 mg/kg) or vehicle (olive oil) was administered orally (0.3 ml) 1 h before injection of carrageenan (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paw. The volumes of the injected and contralateral paws were measured at 1, 3, and 5 h after induction of edema by using a plethysmometer (Ugo Basile, Comerio, Italy). The volume of edema was expressed for each animal as the difference between the carrageenan-injected and contralateral paws. After the last determination of paw edema (5 h), the animals were killed by cervical dislocation, and the right hind paws were homogenized in 2 ml of saline. Aliquots of supernatants were used to determine PGE₂ and LTB₄ as above.

**Rat Air Pouch.** The air pouch procedure was performed in male Wistar rats (120–150 g), as previously described (Edwards et al., 1981). Six days after the initial air injection, 1 ml of sterile saline or 1 ml of 1% w/v zymosan in saline was injected to the air pouch. In the 8-h zymosan-stimulated air pouch, bolinaquinone at 6.3 mg/kg was administered orally in olive oil 30 min before zymosan injection. After 8 h, the animals were killed by cervical dislocation, and the exudate in the pouch was collected with 1 ml of saline. Leukocytes present in exudate in the pouch were measured using a Coulter counter. After centrifugation of exudates (1,200 g at 4°C for 10 min), the supernatants were used to measure PGE₂ and LTB₄ as above. Secretory phospholipase A₂ activity was determined in supernatants previous purification by acid treatment (Bolognese et al., 1995). Aliquots of supernatants were subjected to acid extraction by exposure to equal volumes of 0.36 N H₂SO₄ for 1 h at 4°C. After treatment, the samples were readjusted to pH 7.4 by addition of 2 M Tris, pH 10, the precipitate was removed by centrifugation at 10,000 g for 5 min at 4°C. Supernatants were incubated at 37°C for 15 min in the presence of 10 µl of autoclaved [³H]oleate-labeled membranes of *E. coli* (Franson et al., 1974).

**Adjuvant-induced Arthritis.** Adjuvant arthritis was elicited in female Lewis rats (175–200 g) by injection of 0.1 ml of *M. butyricum* (10 mg/ml) in mineral oil into the base of the tail (Taurog et al., 1988). Paw volumes were measured at the beginning of the experi-

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**Fig. 3.** Topical effect of bolinaquinone and indomethacin on the mouse ear edema model induced by TPA. Data represent the mean ± S.E.M. (n = 6). *, p < 0.05; **, p < 0.01 with respect to TPA control group. Test compounds (µg/ear) were applied topically in acetone 30 min before TPA administration. A, ear edema; B, PGE₂ levels in ear homogenates; C, LTB₄ levels in ear homogenates; D, TNF-α in ear homogenates.

**Fig. 4.** Antiedematogenic effect of bolinaquinone, dexamethasone, and indomethacin by oral route on the mouse ear edema model induced by TPA. Data represent the mean ± S.E.M. (n = 6). **, p < 0.01 with respect to TPA control group. Test compounds (in milligrams per kilogram) were administered p.o. 1 h before TPA administration. Dexa, dexamethasone; Indo, indomethacin.
Fig. 5. Effect of bolinaquinone and indomethacin on the carrageenan mouse paw edema model. Data represent the mean ± S.E.M. (n = 6). *, p < 0.05; **, p < 0.01 with respect control group. Test compounds (in milligrams per kilogram) were administered p.o. 1 h before the injection of carrageenan. A, paw edema at 1, 3, and 5 h after induction of inflammation; B, PGE$_2$ levels in paw exudates; C, LTB$_4$ levels in paw exudates.
Inhibitory effect of bolinaquinone administered orally 30 min before zymosan (6.3 mg/kg) on cellular migration, PGE2 production, LTB4 production, and sPLA2 activity in the 8-h zymosan-injected rat air pouch model. Data represent percentages of inhibition and the mean ± S.E.M. for n experiments. IC50 values were calculated from at least four significant concentrations (n = 6). Statistical evaluation included one-way analysis of variance followed by Dunnett’s t test for multiple comparisons.

Effect on Synthesis and Release of LTB4 and Other Enzyme Activities in Vitro. We first tested the in vitro effect of bolinaquinone on various enzymes involved in the synthesis of inflammatory mediators, such as 5-LO, COX-2, and iNOS. As shown in Fig. 2, bolinaquinone inhibited in a concentration-dependent manner LTB4 generation and release in A23187-stimulated human neutrophils, as well as 5-LO activity in high-speed supernatants from human neutrophils, with IC50 values of 2.1 and 1.3 μM. The reference compound ZM 230,487, a selective inhibitor of 5-LO, showed an IC50 value of 0.1 μM. In addition, as indicated in Table 1, bolinaquinone at 1 μM, coincubated with zymosan, reduced PGE2 and nitrite accumulation around 50% in culture medium from zymosan-stimulated mouse peritoneal macrophages with a lesser potency than the reference compound dexamethasone. It is noteworthy that bolinaquinone caused neither cellular toxicity on human neutrophils nor on mouse peritoneal macrophages at the concentrations used, as determined by reduction of MTT to formazan (data not shown).

Effect on Mouse Ear Edema. Bolinaquinone inhibited potently mouse ear edema induced by TPA after topical (Fig. 3A) and oral administration (Fig. 4). The effect was dose-related with an approximated inhibitory dose 50% (ID50) of 76.7 μg/ear for topical administration and 5.6 mg/kg for oral administration. The topical inflammatory response also showed high levels of PGE2, LTB4, and TNF-α in the ear homogenates of control animals treated with TPA (Fig. 3, B–D). Treatment with bolinaquinone resulted in a significant decrease in PGE2, LTB4, and TNF-α levels and was more effective than indomethacin in this model.

Effect on Mouse Paw Edema. Intraplantar injection of carrageenan to mice caused an inflammatory reaction. Oral pretreatment (1 h before carrageenan) with 3.1, 6.3, 12.5, or 25 mg/kg bolinaquinone reduced hind paw swelling (Fig. 5A). This inhibitory effect was observed at the three time points considered and was as effective as indomethacin in this model. The last evaluation of edema (5 h) was followed by killing the animals, and the paws injected with carrageenan were homogenized to determine the levels of PGE2, PGE2, LTB4, and TNF-α levels as above. Stomachs were homogenized in 2.0 ml of methanol and aliquots of supernatants were used to determine the content of PGE2.

Western Blot Assay. The supernatants from homogenized and centrifuged paws were sonicated and centrifuged at 10,000g for 15 min at 4°C. Supernatant protein was determined, and 25 μg of protein was loaded on 12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in phosphate-buffered saline-Tween 20 containing 3% w/v unfatted milk. For iNOS, membranes were incubated with anti-iNOS polyclonal antibody (1:1,000 dilution); for COX-2, membranes were incubated with specific anti-COX-2 polyclonal antiserum (1:1,000 dilution). Blots were washed and incubated with peroxidase-conjugated goat anti-rabbit IgG (1:20,000 dilution). The immunoreactive bands were visualized using an enhanced chemiluminescence system.

Statistical Analysis. The results are shown as mean ± S.E.M. for n experiments. IC50 values were calculated from at least four significant concentrations (n = 6). Statistical evaluation included one-way analysis of variance followed by Dunnett’s t test for multiple comparisons.

Results
paw but did not modify PGE$_2$ in serum and stomach homogenates as it did with dexamethasone. Nevertheless, the stomachs of the animals treated with dexamethasone showed redness, which was absent in the rats treated with bolinaquinone. Interestingly, bolinaquinone was very effective reducing nitrite and TNF-α in serum and in paws (Fig. 8, B and C).

**Effect on iNOS and COX-2 Protein Expression in Arthritic Paws.** We examined whether the decreased levels in nitrite and PGE$_2$ levels in paw homogenates from bolinaquinone arthritic rats were associated with the inhibition of iNOS and COX-2 expression. As shown by Western blot (Fig. 9), treatment with bolinaquinone potently reduced iNOS protein expression without affecting COX-2 expression measured in supernatants of homogenated arthritic paws on day 25, with respect to the control group. As expected, dexamethasone inhibited the protein expression of both enzymes.

**Radiographic Analysis of the Effect of Bolinaquinone on Adjuvant Arthritis.** A radiographic examination of hind paws from rats 25 days after adjuvant injection revealed bone matrix resorption and osteophyte formation at the joint margin (Fig. 10A) in the arthritic control group. Bolinaquinone and dexamethasone markedly reduced the degree of bone resorption, soft tissue swelling, and osteophyte formation (Fig. 10, B and C).
Prostaglandins, leukotrienes, and nitric oxide are ubiquitous mediator systems exerting numerous vascular and inflammatory effects. Group II secretory PLA_2 can act as a signaling agent contributing to the inflammatory response. This enzyme secreted at inflammatory sites becomes associated with cell surfaces and hydrolyzes phospholipids, thus releasing arachidonic acid, which enters the cell and participates in the increased generation of inflammatory lipid mediators like prostaglandins and leukotrienes (Balsinde et al., 1999).

Previous results indicate that bolinaquinone is a novel inhibitor of human synovial PLA_2 with a higher potency for...
for radiographic examination. The figure is representative of three similar experiments (n = 6 animals/group).

this enzyme than the reference inhibitor manoalide (Gianinni et al., 2001). In addition, bolinaquinone is chemically very close to other molecules, such as avarol and avarone (Ferrándiz et al., 1994), which have been reported as potent anti-inflammatory agents. Notably, bolinaquinone, sharing with avarol and avarone a very similar sesquiterpenoid substructure moiety, differs in the benzenoid part attached to the sesquiterpene moiety. In this regard, bolinaquinone simultaneously contains in its structure the hydroquinone system present in avarol and the quinone system of avarone, which can confer to this compound some of its pharmacological properties.

Our in vitro results indicate that bolinaquinone is able to reduce LTB₄ production in human neutrophils, as well as NO and PGE₂ production in murine macrophages. The chemical structure of bolinaquinone, which contains a hydroquinone/quinone system with a low redox potential, is consistent with the notion that most lipoxygenase inhibitors are able to participate in redox reactions (Ford-Hutchinson et al., 1994; Silverman and Drazen, 1999). In this regard, it has been reported that although redox properties are essential for 5-LO redox inhibitory activity, the lipophilicity plays a dominant role in determining potency for this type of inhibitors (McMillan and Walker, 1992). It is noteworthy that the sesquiterpenoid substructure present in bolinaquinone confers on it a high lipophilicity. We have shown that this effect on LTB₄ production is due to an enzymatic inhibition of 5-LO. Besides, bolinaquinone acted very potently as a topical or oral anti-inflammatory agent. The production of eicosanoids derived from the COX and 5-LO pathways was reduced by this marine product in different models of acute inflammatory response, such as mouse ear edema, carrageenan paw edema, and rat air pouch. This effect is, at least partly, a consequence of reduction in arachidonic acid availability through sPLA₂ inhibition. Interestingly, bolinaquinone also inhibited group II sPLA₂ in vivo in the 8-h zymosan-injected air pouch at a dose that did not affect neutrophil accumulation into the inflammatory exudate. The high reduction observed in LTB₄ production would be a consequence of the dual inhibition of group II sPLA₂ and 5-LO enzymatic activities. A high level of group II sPLA₂ is present in inflammatory fluids and serum of patients from inflammatory diseases (Abe et al., 1997; Fourcade et al., 1998), and thus, the inhibition of sPLA₂ could control the excessive production of lipid mediators and exert protective effects in inflammatory disorders.

The model of adjuvant-induced arthritis in Lewis rats has been used for many years for evaluation of antiarthritic/anti-inflammatory agents (Winder et al., 1969) and is well characterized. In this model, bolinaquinone significantly affected the levels of PGE₂ in the inflamed paw tissue during chronic inflammation, but it did not modify the content of this eicosanoid in serum or stomach. Thus, this marine compound unlike dexamethasone, selectively reduces PGE₂ abnormally elevated during inflammation without reduction of protective levels in other tissues. Besides, bolinaquinone exhibited an important protection against body and spleen weight loss, unlike dexamethasone. In this model, rats develop a chronic swelling in multiple joints, with influx of inflammatory cells, erosion of joint cartilage and bone destruction of joint integrity and loss of function. This model of chronic inflammation is a complex response involving different mediators, and there is therefore a possibility of multiple interactions. In this regard, inflammatory cytokines or iNOS have a role in the development of inflammation (Stefanovic-Racic et al., 1993; Connor et al., 1995; Kollias et al., 1999).

Our data indicate that bolinaquinone is effective, by oral route, in the treatment of experimental chronic inflammation and that the inhibition of joint inflammation was accompanied by reduction of PGE₂, NO, and TNF-α levels. In this regard, cytokines such as TNF-α and IL-1β are inducers of bone and cartilage destruction in the rheumatoid synovium, leading to the therapeutic application of anticytokine therapies in rheumatoid arthritis (Feldmann, 2002). TNF-α can stimulate the release of other cytokines, including IL-1β and chemokines, as well as the expression of adhesion molecules and inducible enzymes (Arias-Negrete et al., 1995). Our results suggest that reduction of NO production might be the consequence of iNOS protein expression inhibition, whereas PGE₂ reduction, which is not due to an inhibition of COX-2 protein expression, would be related with the sPLA₂ inhibitory activity of bolinaquinone, which would limit the bio-

Fig. 9. Effect of bolinaquinone (6.3 mg/kg) and dexamethasone (2 mg/kg) on iNOS and COX-2 expression in homogenized rat paws from adjuvant-induced arthritic rats. Compounds were administered twice daily on days 17 to 24, and paw tissues were recovered on day 25 after adjuvant injection for analysis. The figure is representative of three similar experiments (n = 6 animals/group).

Fig. 10. Effect of bolinaquinone (6.3 mg/kg) and dexamethasone (2 mg/kg) on the radiographic progression of adjuvant-induced arthritis in the tibiotarsal joint of rats. Compounds were administered twice daily on days 17 to 24, and paw tissues were recovered on day 25 after adjuvant injection for radiographic examination. The figure is representative of three similar experiments (n = 6 animals/group).
availability of arachidonic acid. The potent reduction in TNF-α content, exerted by bolaquinone, seems to be based on the complex mechanism of action of this marine product. Whether bolaquinone inhibits inducible NO synthase gene expression, as well as TNF-α production by interaction of nuclear factors or by other mechanisms, remains to be determined.

In summary, we have shown that bolaquinone exerts inhibition of cytokine generation with a characteristic inhibitory profile on iNOS expression and eicosanoid production. The multipronged attack on inflammation and potency of this marine metabolite may have relevance for the inhibition of the inflammatory response and might be of potential interest in the search for new anti-inflammatory agents.

References


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