Introduction

Chronic pain is an aggravating factor that decreases the quality of life of patients with a wide variety of diseases, including direct nerve injury (1). Current therapies for reducing chronic pain often have limited effectiveness and some drugs produce negative side effects (2). Since a variety of molecules involved in the pathological processes of chronic pain have been proposed as analgesic candidates (3–5), alternative drugs with new therapeutic mechanisms have been awaited.

Glycyrrhetinic acid (GA), an aglycone of glycyrrhizin, isolated from the licorice root (Glycyrrhiza), and its semi-synthetic derivatives have a wide range of pharmacological effects (6–8). Previously, we reported that in rodents the GA derivatives, 3β-hydroxy-olean-11,13(18)-dien-30-O-hemiphthalate (Compound 3, Fig. 1) and the disodium salt of olean-11,13(18)-dien-3β,30-O-dihemiphthalate (Compound 5, Fig. 1), inhibited edema formation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) (9), arachidonic acid (10), picryl chloride (11), carrageenan (12), capsaicin, and substance P (SP) (13). Compound 5 also inhibited the writhing response to acetic acid in mice (14). These results suggest that Compound 5 may serve as a novel analgesic agent, although the site of action as well as the analgesic effects of these compounds are still unclear.

On the other hand, it is well known that GA and its derivative, carbenoxolone are gap junction inhibitors and that they show analgesic effects (15–17). From our previous studies, GA and carbenoxolone did not show inhibitory effects in these models, but Compound 5 was inhibitory (9–13). Therefore, we expected that the site of action of Compound 5 is different from that of GA or carbenoxolone.

Mammalian tachykinins comprise a family of peptides with a common carboxyl terminal amide motif (18). SP, a representative family member, is mainly distributed in the peripheral nervous system (PNS) and the central nervous system (CNS), and is involved in nociception and gastrointestinal motility (18–20). The tachykinins act through three major tachykinin receptors, NK1, NK2, and NK3, which are expressed on sensory nerves, immune cells, and in the CNS (21).

To investigate whether GA derivatives may be used as a new class of analgesics, we examined the effects of these compounds on human tachykinin receptors expressed in CHO-K1 cells. Among the GA derivatives examined, the disodium salt of olean-11,13(18)-dien-3β,30-O-dihemiphthalate inhibited the mobilization of [Ca2+]i induced by substance P, neurokinin A, and neurokinin B in CHO-K1 cells expressing the human NK1, NK2, and NK3 tachykinin receptors, respectively. In an inflammatory pain model, Compound 5 suppressed the capsaicin-induced flinching behavior in a dose-dependent manner. Compound 5 was also effective in suppressing pain-related behaviors in the late phase of the formalin test and reducing thermal hyperalgesia in the neuropathic pain state caused by sciatic nerve injury. Collectively, Compound 5 may be an analgesic candidate via tachykinin receptor antagonism.

Keywords: glycyrrhetinic acid, inflammatory pain, neuropathic pain, substance P, tachykinin receptor
nervous system (CNS) and serves as a neurotransmitter in pain perception (19). SP is expressed in nociceptive dorsal root ganglion (DRG) neurons with small cell bodies and is released upon stimulation. The released SP activates its innate receptor neurokinin (NK)₁ on second-order neurons in the spinal cord and transmits intense noxious signals to the higher CNS (20, 21). Studies using knockout mice and selective NK₁-receptor antagonists have revealed that the SP–NK₁ system contributes to a variety of pathological pain states, including inflammatory and neuropathic pain (22). In inflammatory pain, FK888, an NK₁-receptor antagonist, decreased the flinching behavior induced by formalin (23). In neuropathic pain, which is characterized by severe chronic pain caused by damage to the PNS or CNS (1, 2, 24), SP and NK₁ receptor play a crucial role in spinal neuron sensitization through modulation of NMDA-receptor gating (25). However, so far the development of oral NK₁-receptor antagonists has been unsuccessful in relieving clinical pain in humans (26).

In this study, we further characterized the pharmacological profiles of the GA derivatives, including Compound 5, to assess whether they constitute a new class of analgesic agents. Because a previous study showed that Compound 5 is unlikely to bind receptors for histamine, serotonin, prostaglandin, and bradykinin with a high-affinity (12), we hypothesized that this compound may bind to tachykinin receptors and therefore examined its binding potential using cell lines expressing tachykinin receptors. Furthermore, we examined the effects of Compound 5 on hyperalgesia using several behavioral rat pain models.

Materials and Methods

Drugs

GA and its derivatives were prepared at Minophagen Research Laboratory according to the method of Shibata et al. (6) who also reported the physicochemical data for these compounds. The following compounds were used in this study (Fig. 1): GA (11-oxo-olean-12-en-30-oic acid), 18β-olean-11,13(18)-dien-3β,30-diol, 3β-hydroxy-olean-11,13(18)-dien-3β-hemiphthalate, 30-hydroxy-olean-11,13(18)-dien-3β-hemiphthalate, and the disodium salt of olean-11,13(18)-dien-3β,30-dihemiphthalate. SP, NKA, NKB, capsaicin, and complete Freund’s adjuvant (CFA) were purchased from Sigma (St. Louis, MO, USA). Formalin was obtained from Wako (Osaka). SR140333, SR48968, and SR142801 (NK₁-, NK₂-, and NK₃-receptor antagonists, respectively) were generous gifts from Sanofi-Aventis (Paris, France). Compounds 1 – 5 were dissolved in saline containing 0.1% Tween-80 or DMSO. The solvent in the same composition (vehicle) was used as a control. SP, NKA, NKB, and capsaicin were dissolved in saline.

Animals

Male Sprague-Dawley rats (Japan SLC, Inc.,
Hamamatsu) were used at the age of 6 weeks. Rats were kept in an environmentally controlled room at 23 ± 1°C and allowed free access to food and water. This study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. All experimental procedures were also approved by the Nippon Medical School Animal Care and Use Committee (Approval number 21-036).

Preparation of CHO-K1 cells expressing tachykinin receptors

CHO-K1 cells were donated by Riken Cell Bank (Tsukuba) and cultured in F-12 medium containing 10% heat-inactivated fetal calf serum (Nippon Bio-Supp. Center, Tokyo), at 37°C in a humidified atmosphere of 5% CO₂. Culture medium was replaced twice a week. CHO-K1 cells were stably transfected with full length cDNA of human NK₁, NK₂, or NK₃ receptors in pcDNA3.1(+) (Life Technologies, Carlsbad, CA, USA) using Lipofectamine 2000 (Life Technologies). Clones stably expressing each gene were selected by growth in F-12 medium containing 800 μg/ml geneticin (Sigma).

Ca²⁺ mobilization assay

Calcium kit-Fluo 3 (Dojindo, Kumamoto) was used to determine changes in [Ca²⁺], mobilization following the manufacturer’s instructions. CHO-K1 cells stably transfected with human NK₁, NK₂, or NK₃ receptors were plated on a 96-well plate at 8,000 cells per well and cultured overnight. Cells were then loaded with the fluorescent calcium indicator Fluo 3-AM (5 μg/ml) in loading buffer containing 1.25 mM probenecid and 0.04% cremophor EL for 1 h at 37°C. The buffer was then replaced with recording buffer containing 1.25 mM probenecid, Compounds 1 – 5 or tachykinin receptor antagonists, and incubated at 37°C for 20 min. Cells were then treated with SP, NKA, or NKB (1 μM) and changes in fluorescence (excitation at 485 nm and emission at 535 nm) were monitored with a fluorometric plate reader (Wallac 1420 ARVO fluoroscan; PerkinElmer, Waltham, MA, USA) before and after each tachykinin application. To determine the amplitude of the [Ca²⁺] elevation, the mean fluorescent intensity (in arbitrary units) between −0.5 and 0 min prior to compound application was subtracted from the peak intensity in response to each compound. Vehicle was applied to the well as a control. IC₅₀ values were calculated with GraphPad Prism (GraphPad Software, San Diego, CA, USA) using the sigmoidal dose–response function.

Capsaicin-induced spontaneous pain in inflammation model

Capsaicin-induced flinching behavior was induced in inflamed rats as previously described (27). To induce inflammation, 10 μl of CFA was subcutaneously injected into the dorsal surface of the left (ipsilateral) hind paw with a 28-gauge needle. On day 2 after CFA injection, 10 μl of a 0.1% capsaicin solution was subcutaneously injected into the ipsilateral plantar surface of the hind paw with a 28-gauge needle. A 10-μl aliquot of Compound 5 (12.5, 25, or 50 nmol), SR140333 (1 nmol), or vehicle was subcutaneously injected into the ipsilateral hind paw 10 min before capsaicin injection. Spontaneous nociceptive behavior was videotaped and quantified as the average number of flinches per 5 min in the ipsilateral paw for 10 min after the capsaicin injection.

Formalin test

For the induction of acute pain, we used a model of tissue injury–induced pain with formalin as previously described (28). Compound 5 (25, 50, or 100 mg/kg) was orally administered 45 min before the formalin injection, and 30 mg/kg SR140333 was subcutaneously injected 30 min before the formalin injection. A 50-μl aliquot of 1.85% formalin diluted in saline was injected into the dorsal surface of the ipsilateral hind paw with a 28-gauge needle. The animal was immediately placed in the experimental chamber (25 × 15 × 12 cm) to observe the paw flinching behavior. The behavioral response was observed visually and recorded simultaneously with a video camera. Nociceptive behavior was quantified as the average number of flinches in the injected paw during every 5-min period, up to 75 min after the formalin injection.

Neuropathic pain model induced by chronic constrictive injury

Chronic constrictive injury (CCI) was basically produced as previously described (29), except that 4/0 silk thread was used instead of chromic gut. Briefly, rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). The ipsilateral sciatic nerve was exposed at the mid-thigh level, and four 4/0 silk threads were loosely ligated around the nerve at intervals of approximately 1 mm. The incision was closed with a 4/0 silk suture. The right (contralateral) sciatic nerve was left intact as a control. Seven days after the CCI, when hyperalgesia had developed, rats were orally administered with 50 mg/kg Compound 5 or vehicle.

Thermal hyperalgesia was assessed using a Plantar Test (Ugo Basile, Varese, Italy) before CCI and prior to, 45 min, 3 h, and 6 h after Compound 5 or vehicle administration at day 7 after CCI. Briefly, each rat was placed
on a glass plate with a radiant heat generator (a 55-W halogen reflector bulb) underneath. After an acclimation period, radiant heat was separately applied to either the contralateral or ipsilateral hindpaw pad. The latency of paw withdrawal from the thermal stimuli was measured twice at 5-min intervals and the average value was used.

Data analyses

Values were expressed as the mean ± S.E.M. In the in vitro studies, differences in the fluorescent values between the compound treatments were analyzed by one-way analysis of variance (ANOVA), followed by individual post hoc multiple comparisons (Dunnett’s test). In the formalin test and the inflammatory pain model, differences in the number of flinches between the compound treatments were analyzed by one-way ANOVA, followed by individual post hoc multiple comparisons (Dunnett’s test). In behavioral tests for CCI rats, differences in the latency values before and after the compound treatment and in the threshold values between the rats treated with the compound and the vehicle were analyzed by the unpaired t-test. Values of $P < 0.05$ were considered statistically significant.

Results

Effects of GA derivatives on $[\text{Ca}^{2+}]_{i}$ increases induced by SP, NKA, and NKB

When SP, NKA, and NKB at 0.1 $\mu$M (final concentration) were applied to CHO-K1 cells stably expressing human NK1, NK2, and NK3 tachykinin receptors, respectively, rapid $[\text{Ca}^{2+}]_{i}$ increases were observed as changes in fluorescence and peaked at 6 s ($2.8 \times 10^{3} \pm 2.8 \times 10^{2}$ units for SP, $2.9 \times 10^{3} \pm 3.7 \times 10^{2}$ units for NKA, and $8.2 \times 10^{3} \pm 1.0 \times 10^{2}$ units for NKB; $P < 0.05$ or $P < 0.001$, compared with the corresponding controls; $n = 3 – 4$; Fig. 2: A – C). These $[\text{Ca}^{2+}]_{i}$ increases in response to SP, NKA, and NKB were inhibited by pretreatment with the selective antagonists for the cognate receptors, 1 $\mu$M SR140333 (NK1), 1 $\mu$M SR48968 (NK2), and 1 $\mu$M SR142801 (NK3), respectively ($5.8 \times 10^{2} \pm 1.0 \times 10^{2}$ units for SR140333, $9.4 \times 10^{3} \pm 1.6 \times 10^{1}$ units for SR48968, and $1.8 \times 10^{2} \pm 4.4 \times 10^{1}$ units for SR142801; $P < 0.05$ or $P < 0.001$, compared with the corresponding tachykinin treatments alone; $n = 4$; Fig. 2: A – C). None of the tachykinin agonists, tachykinin antagonists, or Compounds 1 – 5 increased $[\text{Ca}^{2+}]_{i}$ in untransfected CHO-K1 cells (data not shown).

Next, we examined the effects of GA (Compound 1 in Fig. 1) and its derivatives (Compounds 2 – 5 in Fig. 1) on the tachykinin-induced $[\text{Ca}^{2+}]_{i}$ increase in CHO-K1 cells expressing tachykinin receptors. Compound 3 at 10 $\mu$M significantly inhibited the $[\text{Ca}^{2+}]_{i}$ increase induced by NKA (16.0% of the NKA-induced response; $P < 0.001$, $n = 4$; Fig. 2B), but showed little effect on the $[\text{Ca}^{2+}]_{i}$ increase induced by SP (Fig. 2A) and NKB (Fig. 2C). Compound 5 at 10 $\mu$M inhibited the $[\text{Ca}^{2+}]_{i}$ increase induced by all three tachykinins in CHO-K1 cells expressing NK1 receptor (4.1% of the SP-induced response; $P < 0.01$, $n = 4$; Fig. 2A), NK2 receptor (28.1% of the NKA-induced response, $P < 0.01$, $n = 4$; Fig. 2B), and NK3 receptor (25.2% of the NKB-induced response; $P < 0.05$, $n = 4$; Fig. 2C). In contrast, phthalic acid (a substituent on three and/or 30 positions of Compounds 3 – 5) and Compounds 1, 2, and 4 at 10 $\mu$M had little effect on the $[\text{Ca}^{2+}]_{i}$ increase induced by either SP, NKA, or NKB (Fig. 2: A – C).

Because Compound 5 showed the most potent antagonistic effects on tachykinin receptors expressed in CHO-K1 cells, we further examined its dose-dependent effects on the tachykinin-induced $[\text{Ca}^{2+}]_{i}$ increase. Pretreatment with 1 – 10 $\mu$M of Compound 5 inhibited the tachykinin-induced $[\text{Ca}^{2+}]_{i}$ increase in CHO-K1 cells expressing the cognate receptors in a dose-dependent manner (Fig. 2: D – F). IC50 values for Compound 5 were 3.5 $\mu$M for NK1, 5.4 $\mu$M for NK2, and 5.3 $\mu$M for NK3. Further, IC50 values for tachykinin receptor antagonists were as follows: SR140333: 6.6 pM for NK1, SR48968: 9.3 pM for NK2, and SR142801: 1.3 nM for NK3.

Effects of Compound 5 on capsaicin-induced pain in inflamed rats

Zhang et al. (27) reported that an NK1-receptor antagonist injected into the rat hind paw suppressed the increase in the number of capsaicin-induced flinches two days after the CFA injection into the hind paw, indicating the involvement of peripheral NK1 receptor activation. Therefore, we followed their protocol and examined the effect of Compound 5 on pain induced by intraplantar capsaicin in inflamed rats.

Before the capsaicin injection, no flinching behavior was observed. Subcutaneous injection of capsaicin into the hind paw induced flinches in vehicle-treated rats for at least 10 min (Fig. 3). Consistent with the report of Zhang et al. (27), pretreatment with the NK1-receptor antagonist SR140333 (1 nmol) suppressed the capsaicin-induced flinching behavior at both 0 – 5 min (16.3 ± 2.1 for SR140333 vs. 46.8 ± 4.4 for vehicle; $P < 0.01$, $n = 8$; Fig. 3), and 6 – 10 min (13.4 ± 3.8 for SR140333 vs. 36.0 ± 4.6 for vehicle; $P < 0.01$, $n = 8$; Fig. 3). Pretreatment with 12.5 – 50 nmol of Compound 5 attenuated the number of capsaicin-induced flinches in a dose-dependent manner at both 0 – 5 min (19.6 ± 1.9 for 50 nmol Compound 5 vs. 46.8 ± 4.4 for vehicle; $P < 0.01$, $n = 8$; Fig. 3) and 6 – 10 min (14.8 ± 2.4 for 50 nmol Compound 5 vs. 36.0 ± 4.6 for vehicle; $P < 0.01$, $n = 8$; Fig. 3).
Fig. 2. Effects of glycyrrhetinic acid and its derivatives on the activations of NK1, NK2, and NK3 receptors in vitro. A – C) The fluorescent intensity of Fluo 3-AM, a calcium indicator, was measured after SP (A), NKA (B), and NKB (C) applications in CHO-K1 cells expressing human NK1, NK2, and NK3 receptors, respectively. Phthalic acid, Compounds 1 – 5, and tachykinin receptor antagonists (SR140333 for NK1, SR48968 for NK2, and SR142801 for NK3) were applied 20 min before applications of tachykinins. D – F) Dose-dependent effects of Compound 5 on the \([\text{Ca}^{2+}]_i\) increase induced by SP (D), NKA (E), and NKB (F). *\(P < 0.05\) and ***\(P < 0.001\), compared with vehicle treatment; **\(P < 0.01\), ***\(P < 0.001\), and ****\(P < 0.0001\): compared with SP, NKA, or NKB treatment by one-way ANOVA followed by individual post hoc multiple comparisons (Dunnett’s test), n = 7 – 8.
Effect of GA Derivative on NK Receptors and Pain

The IC₅₀ value for Compound 5 was 39.9 nmol.

**Effects of Compound 5 on formalin-induced flinching behavior in rats**

In vehicle-treated rats, intraplantar formalin injection produced early (0 – 5 min) and late (15 – 75 min) phases of increase in the nociceptive flinching behavior (n = 9, Fig. 4A). The flinching behavior reached its peak level between 25 and 40 min after the injection and was hardly observed 75 min after the injection.

Next, we examined the effects of Compound 5 on formalin-induced flinching behavior. In rats orally treated with 25 mg/kg Compound 5, a significant reduction in the number of flinches was observed in the late phase (275.6 ± 23.6 for vehicle vs. 193.1 ± 18.5 for Compound 5 between 25 and 40 min after formalin injection; P < 0.01, n = 9; Fig. 4B), but not in the early phase (38.8 ± 1.1 for vehicle vs. 37.1 ± 6.8 for Compound 5 between 0 and 5 min after formalin injection; P = 0.77, n = 9; Fig. 4A). Subcutaneous injection of 30 mg/kg SR140333 (NK₁-receptor antagonist) suppressed the flinching behavior to the same extent as Compound 5 in the late phase (189.4 ± 16.4 for SR140333 between 25 and 40 min after formalin injection; P < 0.01, n = 9; Fig. 4B). We examined the dose-dependent effect of Compound 5 and found no change in the extent of reduction in the number of flinches between 25 and 100 mg/kg (n = 8 – 9, Fig. 4B).

**Effects of Compound 5 on neuropathic pain**

Before the CCI operation on day 0, the latencies of paw withdrawal from thermal stimulation were 11.1 ± 1.2 and 11.1 ± 0.8 s on the ipsilateral and contralateral sides, respectively (n = 8, Fig. 5). After the CCI operation, the latencies significantly decreased on the ipsilateral side on day 7 (6.2 ± 0.4 s on the ipsilateral side vs. 12.2 ± 1.2 s on the contralateral side, P < 0.001; Fig. 5), confirming the development of thermal hyperalgesia.
Next, we examined the effects of peroral administration of Compound 5 on the thermal hyperalgesia in CCI rats. After Compound 5 administration, the rats showed a partial, but significant, reduction in hyperalgesia at 45 min (8.7 ± 1.5 s for Compound 5 vs. 5.4 ± 0.4 s for vehicle; \( P < 0.05 \), \( n = 4 \); Fig. 5A). The analgesic effect of Compound 5 persisted for at least 3 h (8.3 ± 0.7 s for Compound 5 vs. 6.1 ± 0.5 s for vehicle; \( P < 0.05 \); \( n = 8 \); Fig. 5A) and disappeared after 6 h. On the contralateral side, Compound 5 did not affect the latencies of paw withdrawal from thermal stimuli (\( n = 4 – 8 \), Fig. 5B).

Discussion

The present study shows for the first time that GA derivatives antagonized the action of tachykinin receptors in vitro. Consistent with these effects, Compound 5 suppressed hyperalgesia in several animal models of pain through topical and peroral administrations.

Compound 5 had antagonistic effects on human tachykinin receptors as observed in CHO-K1 cells expressing each human tachykinin receptor. On the other hand, phthalic acid, GA (Compound 1), Compound 2, and Compound 4 showed no antagonistic activity on either tachykinin receptor subtype, consistent with our previous studies on inflammatory animal models (10, 12, 13). Compound 3 showed antagonism only to the NK2 receptor. These results suggest that the substituent on the R2 position (Fig. 1) affects the specificity among the tachykinin receptors and the A-ring of the oleanane skeleton is not involved in antagonistic activity to tachykinin receptors. As the IC50s of Compound 5 for NK1, NK2, and NK3 receptors were comparable, Compound 5 seems to have a low specificity to tachykinin receptor subtypes. In contrast, Compound 3 showed preferential antagonism to the NK2 receptor.

In this study Compound 5 reduced hyperalgesia in several rat models of pain. Zhang et al. (27) showed that injection of CFA into unilateral hindpaw produced a longer-lasting inflammation and an increase in NK1 receptor protein expression two days after the injection in the dorsal root ganglion (DRG) neurons. They also reported that the NK1-receptor antagonist Win 51708, injected into the hind paw, reduced capsaicin-induced paw flinching in the first phase (0 – 5 min) and second phase (6 – 10 min) in inflamed rats, suggesting the involvement of peripheral NK1–receptor activation. Consistent with their results, this study shows that Compound 5 injected into intraplantar region dose-dependently reduced the capsaicin-induced paw flinching behavior in both the first and second phases two days after CFA treatment. The result suggests that the analgesic effect of Compound 5 is mediated by NK1-receptor blockade. If this were the case, Compound 5 would have no species-selectivity as well as subtype-selectivity in the receptor-blocking action, which is often observed with non-peptidergic NK1-receptor antagonists (30).

In the next two pain models, Compound 5 was perorally treated. From our previous results, we detected Compound 5 in mouse serum 60 min after the administration (data not shown). The model of tissue injury-induced pain with formalin injection, adopted in the present study, has been well studied (28, 31). An interesting feature of the formalin-induced pain in rodents is that animals show two phases of increase in pain-related be-
haviors, which are thought to reflect distinct underlying mechanisms. Flinching observed in the early phase (5 min after the formalin injection in this study) is caused probably by formalin-induced direct activation of C fiber afferent nociceptors (28, 32). Behavioral responses in the late phase (25 to 40 min after the formalin injection in this study) are thought to depend on central hypersensitivity of the second-order dorsal horn neurons (spinal sensitization) (33, 34). Like in previous reports using other NK1-receptor antagonists (35, 36), Compound 5 and SR140333 did not block transient or physiological pain observed in the early phase of the formalin response. In contrast, Compound 5 and SR140333 inhibited flinching behavior in the late phase. However, the effect of Compound 5 was not dose-dependent. In this experiment, Compound 5 was administered orally. In our preliminary experiment, we could hardly detect Compound 5 in brain tissue homogenate, although we could detect it in serum, 3 h after the oral administration to rats in a dose of 200 mg/kg (data not shown). From these results, we may interpret that this compound has a central effect, but its penetration into the central nervous system is poor.

Compound 5 also had an analgesic effect on thermal hyperalgesia induced by peripheral nerve injury, although the effect was rather limited. The neuropathic pain state has been speculated to result from a wide range of mechanisms, most of which are still poorly understood (1). Tachykinin systems also play a part in the neuropathic mechanisms, most of which are still poorly understood (1). Like injection of the NK1 receptor antagonist RP67580, increased the withdrawal latency (40).

In conclusion, GA derivatives, especially Compound 5, may be promising candidates for antagonists of tachykinin receptors and for analgesic agents in the inflammatory and neuropathic pain states. GA belongs to the family of oleanane-type triterpenoids, which have a chemical structure completely different from any tachykinin-receptor antagonists previously reported. Therefore, this compound provides a new class of lead compounds with activity as tachykinin receptor antagonists.

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