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Interaction of cyclooxygenase-2 inhibitors and salicylate in gastric mucosal damage

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Abstract

The interactions of sodium salicylate and the selective cyclooxygenase-2 inhibitors *N*-{2-(cyclohexyloxy)-4-nitrophenyl}-methanesulfonamide (NS-398) and 5.5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5II)-furanone (DFU), dexamethasone and the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methylester (L-NAME) were examined in ischaemia–reperfusion damage and adaptive protection in the rat stomach. Ischaemia–reperfusion damage was substantially aggravated by pretreatment with NS-398 (4 mg/kg), DFU (2 mg/kg), dexamethasone (1 mg/kg) or L-NAME (3 and 10 mg/kg). Salicylate (0.01–0.05 mg/kg) reversed the aggravating effect of NS-398, DFU and dexamethasone, while the effect of L-NAME was counteracted by L-arginine (twice 400 mg/kg) but not salicylate (0.05 or 10 mg/kg). Instillation of 20% ethanol prevented mucosal damage induced by 70% ethanol. This adaptive gastroprotection was abolished by pretreatment with NS-398 (1 mg/kg), DFU (0.2 mg/kg) or L-NAME (10 mg/kg). Salicylate (0.01–0.05 mg/kg) reversed the inhibition of protection by NS-398 and DFU, while the effect of L-NAME (10 mg/kg) was antagonized by L-arginine (100 mg/kg) but not salicylate (0.05 mg/kg). The precise mechanism of the functional antagonism between extremely low doses of salicylate and selective cyclooxygenase-2 inhibitors remains to be investigated.

Keywords: Sodium salicylate; Cyclooxygenase-2; Gastric mucosal damage; Ischaemia–reperfusion; Adaptive gastroprotection; Nitric oxide (NO)

1. Introduction

Highly selective inhibitors of cyclooxygenase-2 such as *N*-{2-(cyclohexyloxy)-4-nitrophenyl}-methanesulfonamide (NS-398) (Futaki et al., 1994) and 5.5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5II)-furanone (DFU) (Riendeau et al., 1997) have been shown to prevent adaptive protection and to aggravate ischaemia–reperfusion injury in the rat stomach (Gretzer et al., 1998; Maricic et al., 1999). Pretreatment with low doses of 16,16-dimethyl-prostaglandin E_2 that were not protective when given alone, completely reversed the effects of the selective cyclooxygenase-2 inhibitors on adaptive gastroprotection as well as on ischaemia–reperfusion injury (Gretzer et al., 1998; Maricic et al., 1999). Frolich (1997) has classified salicylate as a selective cyclooxygenase-2 inhibitor. In human A549 cells, sodium salicylate has been shown to be an effective inhibitor of cyclooxygenase-2 activity, which, however, was easily displaced by arachidonic acid (Mitchell et al., 1997). On the other hand, Botting and Vane (1997) have pointed out that in a variety of in vitro experiments no selectivity of salicylate for the cyclooxygenase-2 enzyme could be demonstrated. In fact, sodium salicylate has been found to be a weak inhibitor of platelet thromboxane B_2 formation (Patrignani et al., 1997), which is catalyzed by a typical cyclooxygenase-1 enzyme (Mitchell et al., 1993). Furthermore, inhibition by sodium salicylate of the effects of the non-specific cyclooxygenase inhibitors aspirin and indomethacin on thromboxane B_2 formation by platelets has been described (Cerletti et al., 1981; Dahl et al., 1983), demonstrating affinity of salicylate to the cyclooxygenase-1 enzyme. The possible interaction of sodium salicylate with highly selective cyclooxygenase-2 inhibitors has so far not been described. We have, therefore, investigated the modulation by sodium salicylate of the effects of NS-398 and DFU on adaptive protection and ischaemia–reperfusion injury in the rat stomach. This work was presented in part at the 1999

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summer meeting of the British Pharmacological Society in Nottingham, UK, and was published in abstract form (Peskar and Peskar, 1999).

2. Materials and methods

2.1. Animals

Male Wistar rats (180–220 g) were fasted overnight with free access to tap water. All experimental protocols comply with the European Community guidelines for the use of experimental animals and were approved by the Animal Care Committee of the Ruhr-University of Bochum.

2.2. Ischaemia–reperfusion damage of the gastric mucosa

The rats were anaesthetized (pentobarbital, 50 mg/kg, i.p.), tracheotomized and the stomachs exposed by a midline incision. The pylorus was ligated, the celiac artery clamped and 1 ml of acid (100 mM HCl) injected into the gastric lumen. Reperfusion was established 15 min later by removal of the clamp. After a 30 min reperfusion period, the stomach was excised and gross mucosal damage was assessed in a blinded manner by calculation of a lesion index using a 0–3 scoring system based on the number and length of the lesions as described in detail previously (Stroff et al., 1996).

2.3. Drug treatment

Groups of rats were pretreated with NS-398 (4 mg/kg, s.c.) or DFU (2 mg/kg, s.c.) 30 min prior to induction of ischaemia. The doses of NS-398 and DFU were chosen from previous studies in which they had been shown to substantially aggravate mucosal damage induced by ischaemia–reperfusion (Maricic et al., 1999). These doses of NS-398 and DFU selectively inhibited cyclooxygenase-2 (Futaki et al., 1994; Gretzer et al., 1998; Maricic et al., 1999). Additional groups of rats were pretreated with dexamethasone (1 mg/kg, s.c.) 2 h prior to clamping the celiac artery. This treatment was shown previously to increase mucosal ischaemia–reperfusion damage to a comparable extent as treatment with selective cyclooxygenase-2 inhibitors (Maricic et al., 1999). Further groups of rats were pretreated with the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methylester (L-NAME, 3 or 10 mg/kg, i.v.) 10 min prior to induction of ischaemia. In additional experiments, rats received s.c. injections of sodium salicylate at a dose of 0.05 mg/kg 30 min prior to administration of DFU and dexamethasone and at doses of 0.05 and 10 mg/kg 30 min prior to administration of L-NAME. Further groups of rats were injected i.v. twice with 400 mg/kg L-arginine immediately before administration of L-NAME as well as before the reperfusion period.

To assess the dose-dependency of the salicylate effect, groups of rats subjected to ischaemia–reperfusion were pretreated with graded doses of salicylate (0.01–0.05 mg/kg, s.c.) 30 min prior to administration of NS-398. Finally, some rats received salicylate (0.05 mg/kg, s.c.) without additional drug treatment 60 min prior to induction of ischaemia. Controls treated with the corresponding vehicle were included in all experiments. As the observations were identical in rats treated with the various vehicles, results in control rats were combined.

2.4. Adaptive gastroprotection

Rats received 1 ml of the mild irritant 20% ethanol by oral intubation followed by oral instillation of 1 ml of 70% ethanol 30 min later. Controls received 1 ml of water prior to instillation of 70% ethanol. Five minutes after challenge of the gastric mucosa with 70% ethanol, the rats were killed by cervical dislocation. The stomach was removed and gross mucosal damage was assessed in a blinded manner by calculation of a lesion index as described above.

2.5. Drug treatment

Groups of rats were pretreated with NS-398 (1 mg/kg, p.o.) or DFU (0.2 mg/kg, p.o.) 30 min before instillation of 1 ml of 20% ethanol. The doses of NS-398 and DFU were chosen from previous investigations in which they had been found to abolish the gastroprotective effect of 20% ethanol against mucosal damage induced by 70% ethanol (Gretzer et al., 1998) and to be selective for cyclooxygenase-2 (Futaki et al., 1994; Gretzer et al., 1998; Maricic et al., 1999). Further groups of rats received L-NAME (10 mg/kg, i.v.) 10 min prior to instillation of the mild irritant. In addition, groups of rats were treated with graded doses of salicylate (0.01–0.05 mg/kg, p.o.) 30 min prior to administration of NS-398. In further experiments, salicylate (0.05 mg/kg, p.o.) was administered 30 min prior to DFU or L-NAME. Other groups of rats received L-arginine (100 mg/kg, i.v.) immediately before administration of L-NAME. To evaluate whether salicylate at the dose used has gastroprotective activity by itself, the drug (0.05 mg/kg, p.o.) was administered 30 min prior to oral instillation of 1 ml of water followed by challenge of the gastric mucosa with 70% ethanol 30 min later. Controls treated with the corresponding vehicle were included in all experiments. As the observations were identical in rats treated with the various vehicles, results obtained in control rats were combined.

Previous studies have shown that pretreatment with dexamethasone did not diminish the protection induced by 20% ethanol (Gretzer et al., 1998). Therefore, the effect of salicylate in dexamethasone-treated rats was not studied in the mild irritant gastroprotection model.

2.6. Statistical analysis

All data are expressed as means ± S.E.M. of n values. Comparisons between groups were made using the Wil-

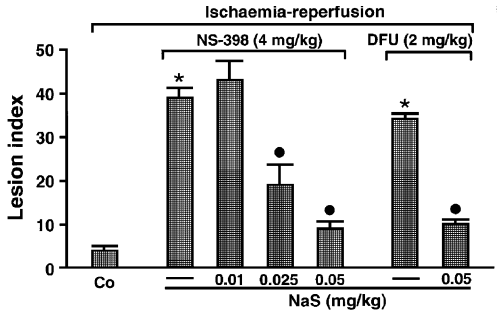

Fig. 1. Increase in gastric mucosal damage induced by ischaemia–reperfusion by cyclooxygenase-2 inhibitors and the effect of salicylate. Pretreatment with NS-398 (4 mg/kg, s.c.) or DFU (2 mg/kg, s.c.) aggravated mucosal damage elicited by ischaemia–reperfusion. Salicylate (NaS, 0.01–0.05 mg/kg, s.c.) reversed the effect of NS-398 and DFU. Values are the means ± S.E.M. of 4–10 rats. As the various vehicles did not modify mucosal damage induced by ischaemia–reperfusion, values of vehicle-treated controls were combined ($n = 12$); * $P < 0.001$ vs. ischaemia–reperfusion alone; ● $P < 0.001$ vs. NS-398 or DFU alone.

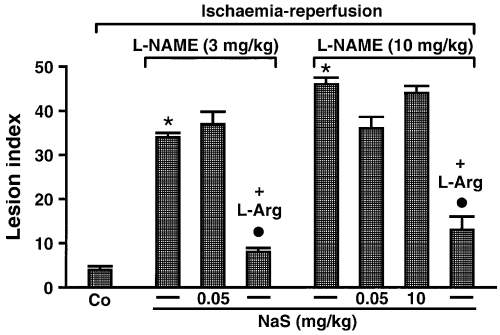

Fig. 3. Increase in gastric mucosal damage induced by ischaemia–reperfusion by NO synthase inhibition and the effect of salicylate and L-arginine. Pretreatment with L-NAME (3 and 10 mg/kg, i.v.) augmented gastric mucosal damage in a dose-dependent manner. L-arginine (L-Arg, twice 400 mg/kg, i.v.) but not salicylate (NaS, 0.05 or 10 mg/kg, s.c.) reversed the effect of L-NAME. Values are the means ± S.E.M. of four to seven rats. * $P < 0.001$ vs. vehicle-treated controls; ● $P < 0.001$ vs. L-NAME alone.

coxon rank test. A probability of $P < 0.05$ was considered as statistically significant.

2.7. Materials

DFU was a generous gift from Dr. A.W. Ford-Hutchinson (Merck-Frosst, Montreal, Canada). NS-398 was from Cayman Chemical (Ann Arbor, MI, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

DFU was dissolved in 50 μl of methylsulfoxide/Tween 80 (1:1 by volume) and further diluted 1:20 in saline containing 1% Tween 80. NS 398 was dissolved in absolute ethanol (5 mg/ml) and further diluted in saline containing 1% Tween 80. L-NAME and sodium salicylate were dissolved in saline. Dexamethasone was suspended in methylcellulose (0.25%). Drugs were administered in a volume of 2.5 ml/kg.

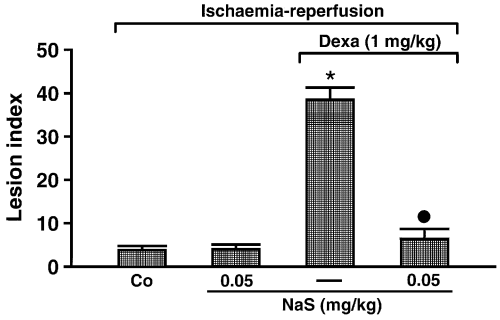

Fig. 2. Increase in gastric mucosal damage elicited by ischaemia–reperfusion by dexamethasone and the effect of salicylate. Pretreatment with dexamethasone (Dexa, 1 mg/kg, s.c.) markedly aggravated mucosal damage. Salicylate (NaS, 0.05 mg/kg, s.c.) reversed the effect of dexamethasone. Salicylate (0.05 mg/kg, s.c.) given alone did not affect ischaemia–reperfusion-induced damage. Controls received the vehicle ($n = 10$). Values are the mean ± S.E.M. of 4–10 rats. * $P < 0.001$ vs. vehicle-treated controls; ● $P < 0.001$ vs. dexamethasone alone.

3. Results

3.1. Effect of salicylate in rats with gastric ischaemia–reperfusion damage

Ischaemia–reperfusion alone induced only minor damage to the gastric mucosa (lesion index 4 ± 1, $n = 12$) that was not affected by pretreatment with the various vehicles

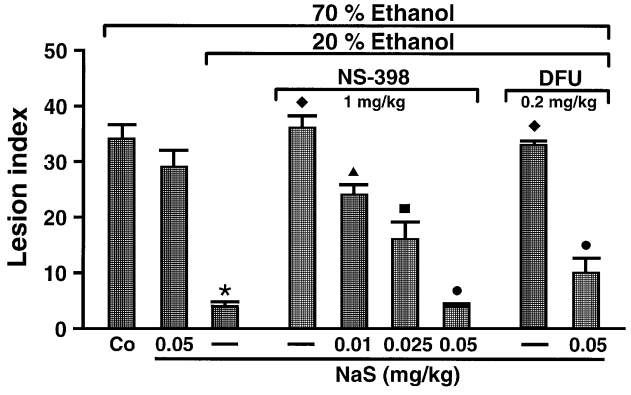

Fig. 4. Inhibition of mild irritant-induced gastroprotection by cyclooxygenase-2 inhibitors and the effect of salicylate. Instillation of 20% ethanol protected against gastric mucosal damage induced by instillation of 70% ethanol. Pretreatment with NS-398 (1 mg/kg, s.c.) or DFU (0.2 mg/kg, s.c.) abolished the protective effect of 20% ethanol. Salicylate (NaS, 0.01–0.05 mg/kg, s.c.) reversed the effect of NS-398 and DFU. Salicylate (0.05 mg/kg, s.c.) given alone did not protect against the injurious effect of 70% ethanol. Values are the means ± S.E.M. of four to eight rats. * $P < 0.001$ vs. vehicle-treated controls, ◆ $P < 0.001$vs. 20% ethanol before 70% ethanol; ▲ $P < 0.02$, ■ $P < 0.01$, ● $P < 0.001$ vs. NS-398 or DFU alone.

used in the experiments. Treatment with NS-398 (4 mg/kg, s.c.) increased ischaemia–reperfusion damage 10-fold ($P<0.001$, $n=10$). Pretreatment with salicylate (0.01–0.05 mg/kg, s.c., $n=4$ each group) reversed the effect of NS-398 significantly and in a dose-dependent manner (ID_{50}: 0.02 mg/kg). Furthermore, ischaemia–reperfusion injury was substantially aggravated in rats pretreated with DFU (2 mg/kg, s.c., $P<0.001$, $n=6$). Administration of salicylate (0.05 mg/kg, s.c.) significantly inhibited by 80±3% the increase in damage evoked by DFU ($P<0.001$, $n=4$). Results are shown in Fig. 1.

Severe mucosal injury was also observed in rats treated with dexamethasone (1 mg/kg, s.c., $n=10$) prior to ischaemia–reperfusion. The damage-aggravating effect of dexamethasone was inhibited by 95±4% in rats pretreated with salicylate (0.05 mg/kg, $P<0.001$, $n=5$). Salicylate (0.05 mg/kg, s.c.) given alone 60 min prior to clamping the celiac artery had no effect on mucosal damage induced by ischaemia–reperfusion (lesion index 4±1, $n=4$). Results are shown in Fig. 2.

L-NAME (3 and 10 mg/kg, i.v.) significantly and dose-dependently augmented ischaemia–reperfusion injury ($P<0.001$ each, $n=5$ and 7, respectively). Pretreatment with salicylate (0.05 mg/kg s.c.) 60 min prior to induction of ischaemia did not reverse the increase in mucosal damage associated with inhibition of NO biosynthesis neither using the high dose of L-NAME (10 mg/kg, $n=5$) that induced widespread mucosal injury nor the submaximally effective dose (3 mg/kg, $n=5$). Even when the dose of salicylate was increased to 10 mg/kg, the aggravation of ischaemia–reperfusion damage induced by L-NAME (10 mg/kg) was not modified ($n=6$). The increase in ischaemia–reperfusion damage induced by L-NAME (3 or 10 mg/kg) was, however, reversed by concurrent treatment with the substrate of NO synthase L-arginine (twice 400 mg/kg, $n=4$ for 3 mg/kg, $n=7$ for 10 mg/kg L-NAME, respectively). Results are shown in Fig. 3.

3.2. Effects of salicylate in rats with mild irritant-induced gastroprotection

Instillation of 20% ethanol significantly reduced by 87± 3% the mucosal damage induced by 70% ethanol. The protective effect of the mild irritant was abolished ($P<0.001$) by pretreatment with NS-398 (1 mg/kg, p.o., $n=6$) or DFU (0.2 mg/kg, p.o., $n=8$). Salicylate (0.05 mg/kg, p.o., $n=5$) given alone did not reduce the damage caused by 70% ethanol but near-maximally counteracted the inhibition of mild irritant-induced protection evoked by DFU ($P<0.001$, $n=6$). Using NS-398 to inhibit mild irritant-induced protection, the salicylate effect was found to be dose-dependent (ID_{50}: 0.02 mg/kg, $n=4–7$ per group). Results are shown in Fig. 4. As illustrated in Fig. 5, L-NAME (10 mg/kg, i.v., $n=5$) completely blocked the protective effect of 20% ethanol. Similar to the observations in the ischaemia–reperfusion damage model, pretreatment with L-arginine (100 mg/kg, i.v., $n=5$) but not with salicylate (0.05 mg/kg, s.c., $n=5$) attenuated the inhibition of mild irritant-induced protection induced by L-NAME.

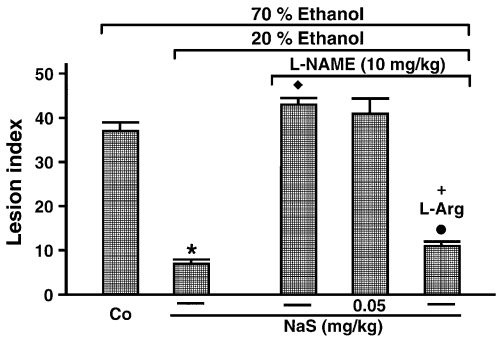

Fig. 5. Inhibition of mild irritant-induced gastroprotection by inhibition of NO synthase. Pretreatment with L-NAME (10 mg/kg, i.v.) abolished the protective effect of 20% ethanol. L-arginine (L-Arg, 100 mg/kg, i.v.) but not salicylate (NaS, 0.05 mg/kg, s.c.) reversed the effect of L-NAME. Values are the means±S.E.M. of five rats. * $P<0.001$ vs. vehicle-treated controls; ◆ $P<0.001$ vs. 20% ethanol before 70% ethanol; ● $P<0.001$ vs. L-NAME alone.

4. Discussion

The present results show that sodium salicylate, like 16,16-dimethyl-prostaglandin E_2, antagonizes the effects of highly selective cyclooxygenase-2 inhibitors on adaptive gastroprotection and ischaemia–reperfusion injury. Remarkably, these effects are achieved with very low doses of sodium salicylate. In fact, no biologically relevant in vivo effects of sodium salicylate have been described so far in the dose range effective in the present experiments. Sodium salicylate has been described to be protective against aspirin and indomethacin-induced gastric injury (Ezer et al., 1976, 1984) as well as against ethanol-induced mucosal damage (Robert, 1981). However, these effects are achieved only with high doses of salicylate. Thus, for protection against ethanol, the ED_{50} is 15 mg/kg (Robert, 1981). The effects described here were observed with doses of sodium salicylate that are about three orders of magnitude lower than those conferring direct gastroprotection. No antagonism of salicylate to L-NAME was observed even with the much higher dose of sodium salicylate excluding non-specific effects of salicylate on gastric protection. It remains to be elucidated whether the modulation by salicylate of the effects of cyclooxygenase-2 inhibitors results from an interaction at the level of arachidonic acid metabolism or is due to a functional antagonism and whether such effects also occur in man.

The interaction of salicylate with highly selective cyclooxygenase-2 inhibitors is reminiscent of the interaction between sodium salicylate and aspirin or indomethacin on platelets. It has been suggested that this drug interaction occurs at a hypothetical supplementary binding site on platelet cyclooxygenase rather than directly on the substrate

active site (Cerletti et al., 1981). In such a way, sodium salicylate, which itself is only weakly active on platelet cyclooxygenase (Patrignani et al., 1997), might inhibit platelet cyclooxygenase inactivation by aspirin and indomethacin (Cerletti et al., 1981; Dahl et al., 1983). In fact, inhibition of generation of thromboxane B_2 and malondialdehyde by aspirin and indomethacin was dose-dependently antagonized by salicylate in vitro and in vivo (Merino et al., 1980; Cerletti et al., 1981; Dahl et al., 1983; Bucchi et al., 1986).

In the experimental models used in the present study, correlation of the biological effects observed with biochemical changes in arachidonic acid metabolism was not possible. It has been pointed out previously that in rat gastric mucosa, cyclooxygenase-2-derived prostaglandins seem to represent only a small fraction of the total prostaglandin pool both under basal conditions in the absence of tissue injury (Gretzer et al., 1998, 2001) and after ischaemia–reperfusion (Maricic et al., 1999). Thus, in gastric tissue, the large amounts of prostaglandins generated via the cyclooxygenase-1 isoform result in a high background that renders isolated assessment of changes of cyclooxygenase-2 activity very difficult. Furthermore, in this situation, the possible modulation of cyclooxygenase-2 inhibition by sodium salicylate could not reliably be determined.

While the effects of highly selective cyclooxygenase-2 inhibitors on adaptive gastroprotection are obviously mediated by inhibition of a constitutive form of the isoenzyme (Gretzer et al., 1998), ischaemia–reperfusion injury results in induction of cyclooxygenase-2 (Kishimoto et al., 1998; Maricic et al., 1999). Consequently, dexamethasone, which inhibits enzyme induction, does not affect adaptive gastroprotection (Gretzer et al., 1998), but aggravates ischaemia–reperfusion injury (Maricic et al., 1999). From our data, it seems possible that in the latter case, salicylate interferes directly with regulation of cyclooxygenase-2 by dexamethasone at the transcriptional level. Alternatively, it may be that the functional antagonism is unrelated to cyclooxygenase-2 or the gastric mucosal prostanoid system. Recently, a number of salicylate effects not directly related to arachidonic acid metabolism has been described. These include inhibition of activation of nuclear factor-κB (Kopp and Ghosh, 1994), activation of p38 mitogen-activated protein kinase (Schwenger et al., 1997), antioxidant properties with particular protection against ischaemia–reperfusion injury (Van Jaarsfeld et al., 1994; Colantoni et al., 1998) and the release of adenosine (Cronstein et al., 1999). We have observed previously (Trautmann et al., 1991) that direct gastroprotective effects of various non-steroidal antiinflammatory drugs including several salicylates are not correlated with specific effects on mucosal cyclooxygenase, 5-lipoxygenase or 15-lipoxygenase activity.

In conclusion, while the antiinflammatory activity of salicylate as well as aspirin has been explained by inhibition of prostaglandin E_2 production by salicylate (Higgs et al., 1987), the exact mechanisms of the protective activity of sodium salicylate in gastric mucosa in general and the antagonism against highly selective cyclooxygenase-2 inhibitors and dexamethasone in particular remain to be further investigated.

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