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Inhibition of Peroxynitrite-Induced Dityrosine Formation with Oxidized and Reduced Thiols, Nitric Oxide Donors, and Purine Derivatives

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ABSTRACT

Peroxynitrite, formed by the combination of superoxide anion and nitric oxide, is a powerful oxidant at physiological pH and is apparently involved in the pathogenesis of several human diseases. Therefore, inhibitors of peroxynitrite-induced oxidation are important targets for pharmaceutical development. The reaction of peroxynitrite with L-tyrosine, one of its biological targets, yields stable products, including nitrotyrosine and dityrosine. Here we test the ability of thiols, nitric oxide donors, and purine derivatives to inhibit peroxynitrite-induced dityrosine formation in a physiological buffer containing bicarbonate/CO_2. We show that both reduced and oxidized thiols, nitric oxide donors, and urate, but not other purine derivatives, reduce peroxynitrite-induced dityrosine formation. Antioxid. Redox Signal. 3, 165–171.

INTRODUCTION

PEROXYNITRITE ($ONOO^-$) is a powerful oxidant species that can be produced *in vivo* by the combination of nitric oxide (NO) and superoxide anion at an extremely rapid rate ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$), which is near the diffusion limit. At physiological pH, peroxynitrite is protonated to form peroxynitrous acid, which rapidly decomposes to form highly reactive oxidant species. The *in vivo* cytotoxic effects of peroxynitrite include lipid peroxidation, nitration of aromatic rings, oxidation of sulfhydryl groups, DNA strand breakage, and inhibition of mitochondrial respiration (for review, see 2), leading to tissue injury, which manifests itself, for example, as a depression in myocardial contractile function (10, 18). As peroxynitrite-induced cytotoxicity is reported to mediate tissue injury in a variety of human diseases, development of peroxynitrite scavengers is of great importance (for review, see 21).

The reaction of peroxynitrite with tyrosine induces nitration of its aromatic ring via intermediate formation of tyrosyl radicals, thereby yielding nitrotyrosine and dityrosine (22). Although there is some controversy as to the mechanism and the specificity of the nitration reaction *in vivo* (8, 13), some of this controversy may be due to the study of peroxynitrite in non-physiological buffer systems. Nitrotyrosine and dityrosine, two stable tyrosine derivatives, are used as markers of peroxynitrite formation in biological systems. The fluorometric assay

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for dityrosine provides a sensitive and low-cost method to estimate peroxynitrite formation (22) and has been successfully applied, for example, to show acute release of peroxynitrite during reperfusion following ischemia in the isolated rat heart (28).

Many of the putative scavengers of peroxynitrite were previously tested in simple phosphate buffer systems. However, bicarbonate/CO_2 is responsible for the majority of the buffering capacity of the extracellular and intracellular fluids in biological systems. CO_2 reacts with peroxynitrite and forms a reactive adduct, the nitrosoperoxycarbonate anion (5, 14). This adduct and reactive products derived from it upon protonation at physiological pH show altered reactivity with biological targets compared with that of peroxynitrite in non-physiological buffer systems in the absence of bicarbonate/CO_2. In the presence of bicarbonate/CO_2 at physiological pH, peroxynitrite causes increased nitration of aromatics, decreased oxidation of thiols, and inhibited hydroxylation of benzoate (5, 19). Indeed, it has been suggested that some reactions attributed to peroxynitrite, such as nitration of tyrosine, are dependent upon nitrosoperoxycarbonate and not peroxynitrite (3).

Thiols (4, 15), NO-generating drugs (16, 28), and uric acid (9, 23) have been shown to reduce peroxynitrite-mediated injury in a variety of biological systems. However, their potency and efficacy have not been comparatively evaluated in physiological bicarbonate/CO_2 buffers at 37°C and pH 7.4, which would better approximate *in vivo* reaction conditions. Therefore, we here tested a panel of these compounds under these conditions and compared their ability to prevent peroxynitrite-mediated oxidation of tyrosine, one of the biological targets of peroxynitrite.

MATERIALS AND METHODS

Chemicals

Adenosine, cysteine, cystine, reduced (GSH) and oxidized glutathione (GSSG), hypoxanthine, xanthine, and urate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). SP/W-6373 [*N*-(3-hydroxypivaloyl)-*S*-(*N'*-acetylglycyl)-L-cysteine ethyl ester], SP/W-5186 [*N*-(3-nitropivaloyl)-*S*-(*N'*-acetylglycyl)-L-cysteine ethyl ester]. SP/W-4744 [3-nitropivalic acid], and *S*-nitrosoglutathione (GSNO) (Fig. 1) were obtained from Schwarz Pharma AG (Monheim, Germany). Krebs–Henseleit solution contained 118 m*M* NaCl, 4.7 m*M* KCl, 1.2 m*M* $MgSO_4$, 1.75 m*M* $CaCl_2$, 0.5 m*M* EDTA, 25 m*M* $NaHCO_3$, 1.2 m*M* KH_2PO_4, 11 m*M* glucose (buffer salts from BDH Inc., Toronto, Ontario, Canada), 0.2% bovine serum albumin (Boehringer Mannheim, Germany), and 0.3 m*M* L-tyrosine (Sigma Chemical Co.), and was bubbled vigorously with carbogen (95% O_2 and 5% CO_2) at 37°C (pH 7.4). Stock solutions of adenosine, cysteine, GSH, GSSG, GSNO, SP/W-6373, SP/W-4744, and SP/W-5186 (18 m*M* each) were dissolved in Krebs–Henseleit solution. Stock solutions of cystine, hypoxanthine, urate, and xanthine (18 m*M* each) were dissolved in alkaline Krebs–Henseleit solution containing 70 m*M* NaOH, and were further diluted using normal Krebs–Henseleit solution.

Synthesis of peroxynitrite

Peroxynitrite was prepared by the method described by Villa *et al.* (24). In brief, an ice-cold aqueous solution of $NaNO_2$ (12 m*M*) and a solution containing nitric acid (11.1 m*M*) and H_2O_2 (8.2 *M*) were taken into each of two syringes (10 ml) connected with a Y-piece tubing and discharged rapidly into a stirring solution of 6.6 ml of ice-cold NaOH (4.2 *M*). Excess H_2O_2 was removed by treating the mixture with 2 g of granular $Mn(IV)O_2$ for 2 min. The solution was then filtered through a Whatman no. 54 filter paper. The concentration of peroxynitrite was determined by ultraviolet spectroscopy ($\lambda_{max} = 302$ nm, $\zeta = 1{,}670\ M^{-1}\ cm^{-1}$) and stored in aliquots at −80°C. The peroxynitrite concentration of the aliquots was checked before each assay, and stock solutions were further diluted with ultrapure water.

Measurement of peroxynitrite-induced dityrosine formation

Reactions were performed in Krebs–Henseleit buffer containing 0.3 m*M* L-tyrosine and 95% O_2/5% CO_2 at 37°C (pH 7.4). We have pre-

viously shown that addition of peroxynitrite to this buffer results in a rapid (<1 min) formation of dityrosine, which can be detected by both fluorescence spectroscopy and HPLC with an excellent linear correlation ($r^2 = 0.98$) between the HPLC and fluorescence-based determinations of dityrosine in the same sample (28). The different test compounds (100 μl volume) were added to microcentrifuge tubes to give a final concentration in 1,800 μl of 0 (vehicle), 30, 100, 300, or 1,000 μM, respectively. An aliquot of the buffer (1,700 μl) was then added to each tube. The tubes were immediately capped, vortexed, and placed into a 37°C water bath for 2 min. Each tube was briefly opened, peroxynitrite (30 μl) was added to give either a 3 or 30 μM final concentration, and then the tube was recapped. Tubes were immediately vortexed for 15 s, incubated for 10 min at 37°C, and then placed on ice. The entire volume of the reaction mixture was transferred into a plastic cuvette for fluorometric assay. To detect dityrosine, samples were excited at 320 nm wavelength and scanned between the emission wavelengths of 360 and 500 nm (scan time 3 s) in a spectrofluorometer (Shimadzu, Model RF 5000). To test if any of the studied compounds may have quenched the fluorescent signal from dityrosine, peroxynitrite was added first to Krebs–Henseleit buffer containing L-tyrosine and incubated for 10 min at 37°C, and the highest concentration of the test compound was then added (at which time peroxynitrite was already decomposed). None of the tested compounds interfered with dityrosine fluorescence (data not shown).

The fluorescent signal amplitude was measured at 411.2 nm, and any background fluorescent signal derived from Krebs–Henseleit buffer containing L-tyrosine was subtracted from this. Inhibition of dityrosine formation by a test compound was calculated as the percentage of the peak amplitude compared with that in the presence of the vehicle.

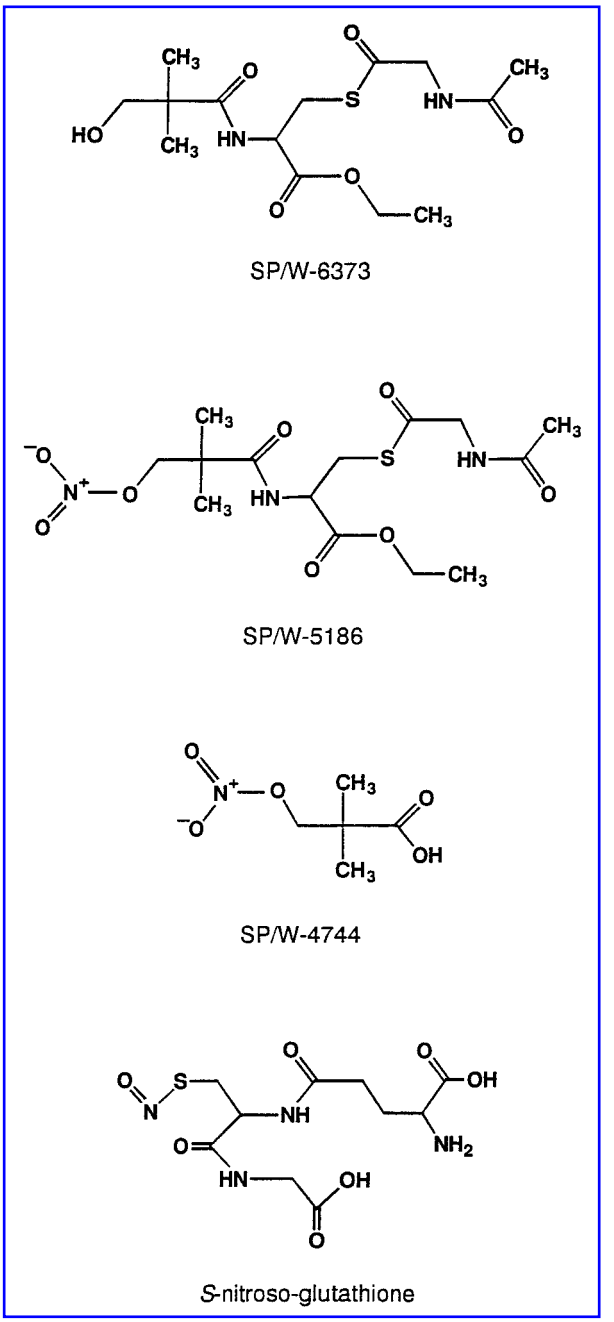

FIG. 1. Structures of SP/W-6373, SP/W-5186, SP/W-4744, and GSNO.

RESULTS

When 3 or 30 μM peroxynitrite was added to Krebs–Henseleit buffer containing 0.3 mM L-tyrosine at physiological pH and temperature, formation of dityrosine was inhibited by both reduced and oxidized thiols (Fig. 2). Figure 2A shows that at 3 μM peroxynitrite, cysteine was more potent than cystine and GSSG was more potent than GSH. Both cysteine and cystine were more potent than GSSG and GSH. Figure 2B demonstrates that the pattern of inhibitory activity of these compounds was somewhat different when a 10-fold higher (30 μM) con-

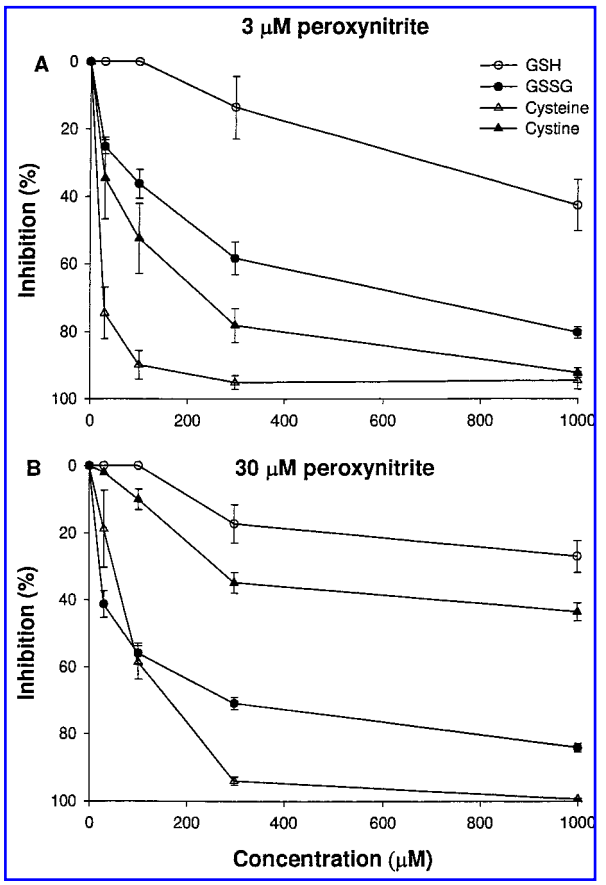

FIG. 2. Inhibitory effects of reduced and oxidized thiols on dityrosine formation induced by 3 μM (A) and 30 μM (B) peroxynitrite in Krebs–Henseleit buffer containing bicarbonate/CO_2 and L-tyrosine at physiological temperature and pH. Results are means ± SEM ($n \geq 3$).

centration of peroxynitrite was used. Again cysteine had a greater potency than cystine and GSSG was more potent than GSH; however, GSSG was a more effective inhibitor than cystine.

Peroxynitrite (3 μM)-mediated dityrosine formation was inhibited by the three NO donor compounds tested (Fig. 3). GSNO was the most effective inhibitor and the cysteinyl containing NO donor SP/W-5186 was the least effective. The control for SP/W-5186, the cysteinyl containing compound without NO donor capacity SP/W-6373, showed an equivalent inhibitory profile to SP/W-5186. The NO donor without cysteinyl moiety SP/W-4744 showed an intermediate effect (Fig. 3A). A similar pattern was seen at 30 μM peroxynitrite, except that low concentrations of SP/W-4744 (30–100 μM) showed a stronger inhibitory effect than the other compounds (Fig. 3B).

Whereas urate exerted a marked inhibitory action on peroxynitrite-induced dityrosine formation, the other purine derivatives (adenosine, hypoxanthine, and xanthine) were without effect (Fig. 4).

DISCUSSION

The oxidative reactions of peroxynitrite show different characteristics in buffers containing bicarbonate/CO_2 when compared with those in other solutions, as peroxynitrite readily reacts with CO_2 to form the nitrosoperoxycarbonate anion (5). Extracellular and intracellular fluids contain bicarbonate (25 mM and 12 mM, respectively), which is in equilibrium with

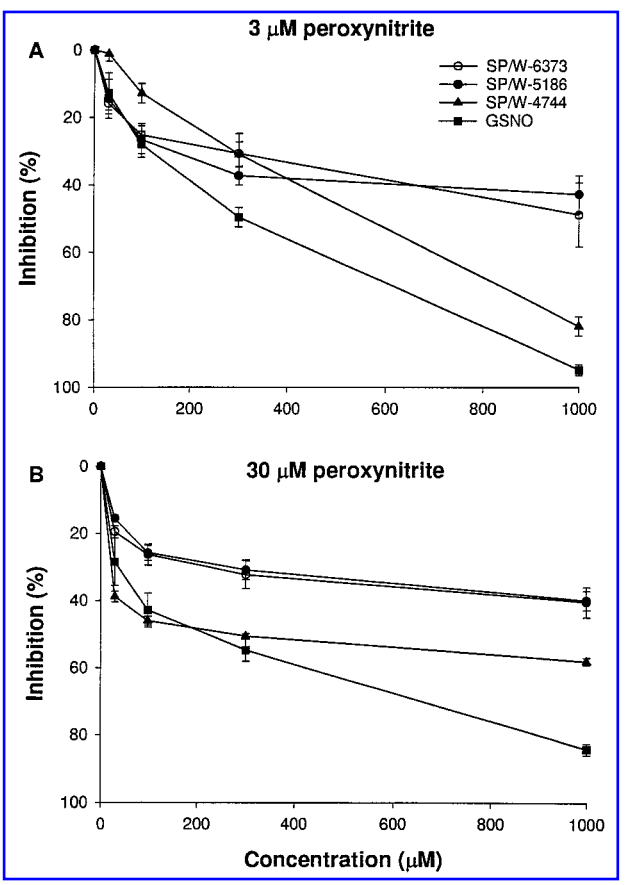

FIG. 3. Inhibitory effects of NO donor compounds without (GSNO, SP/W-4744) or with cysteinyl (SP/W-5186) moiety on dityrosine formation induced by 3 μM (A) and 30 μM (B) peroxynitrite in Krebs–Henseleit buffer containing bicarbonate/CO_2 and L-tyrosine at physiological temperature and pH. Results are means ± SEM ($n \geq 3$).

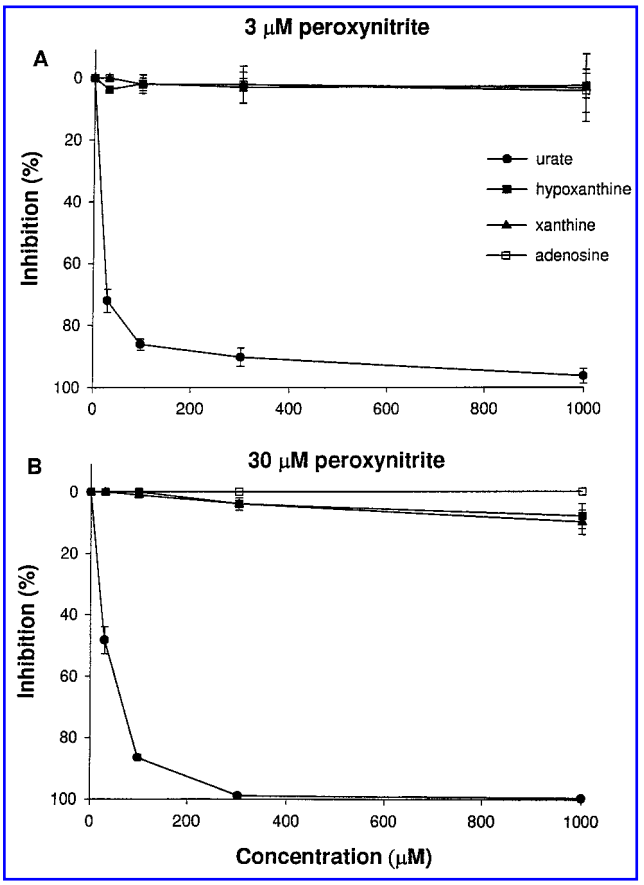

FIG. 4. Inhibitory effects of purine derivatives on dityrosine formation induced by 3 μM (A) and 30 μM (B) peroxynitrite in Krebs–Henseleit buffer containing bicarbonate/CO_2 and L-tyrosine at physiological temperature and pH. Results are means ± SEM ($n \geq 3$).

CO_2. Therefore, we tested the ability of a panel of putative peroxynitrite scavengers to inhibit peroxynitrite-induced dityrosine formation in Krebs–Henseleit buffer containing bicarbonate/CO_2 and L-tyrosine at physiological pH and temperature. Although both nitrotyrosine and dityrosine are products of the reaction between peroxynitrite and tyrosine, we chose to follow production of the latter as dityrosine is fluorescent, providing both better sensitivity and greater selectivity for its detection.

Here we show that in a bicarbonate/CO_2 containing physiological buffer, both reduced and oxidized thiols inhibit peroxynitrite-induced conversion of L-tyrosine to dityrosine. In this system, cysteine was much more potent and efficacious than GSH and either of the thiol's oxidized counterparts. As the concentration of cysteine in human body fluids is ~8–10 μM (1), our data suggest that it may be an effective endogenous scavenger of peroxynitrite. Moreover, in this role GSH would likely be equally important, because its intracellular concentration is 100–1,000-fold higher than that of cysteine (1). Interestingly, GSSG was a more potent inhibitor of dityrosine formation than GSH. In phosphate buffer, a panel of reduced thiols including GSH showed stronger peroxynitrite scavenging effects than their oxidized counterparts, although the disulfide and the thiol form of lipoic acid protected equally against inactivation of α_1-antiproteinase induced by 500 μM peroxynitrite (25, 26). The production of dityrosine from the reaction of peroxynitrite with tyrosine involves the formation of tyrosyl radicals as an intermediate (23). As several thiols are known to generate thiyl-derived radicals upon exposure to peroxynitrite (7, 11), these radicals would compete for tyrosyl radicals, and thus one would predict a reduction in dityrosine formation by thiols as we observed here. Whether peroxynitrite can also generate thiyl radicals from oxidized thiols is unknown.

We also demonstrate here that NO donor molecules exert an inhibitory effect against peroxynitrite-induced formation of dityrosine. Our results demonstrate that the cysteinyl containing compounds with (SP/W-5186) or without (SP/W-6373) NO donor capacity show a weak but equivalent inhibitory profile. This suggests that the NO donor capacity and/or cysteinyl moiety of these compounds provides some protection against peroxynitrite-induced formation of dityrosine. An alternative explanation for the protective action of SP/W-6373 is because of its free hydroxy group, as molecules containing an alcohol functional group have been shown to react with peroxynitrite to form NO donor intermediates (12). Among the NO donor compounds tested her, GSNO was the most potent peroxynitrite scavenger, SP/W-4744 had an intermediate effect, and SP/W-5186 was the weakest. Our results suggest that NO donors may protect against peroxynitrite-induced oxidative processes and that the differences between compounds may be explained in the rate by which they release NO, as GSNO has the greatest release rate of NO of the three NO donors tested here (K. Knuettel, Schwartz Pharma AG, personal com-

munication). This is in accordance with our previous studies in isolated rat heart showing that the NO donor *S*-nitroso-*N*-acetylpenicillamine improved cardiac mechanical function and decreased peroxynitrite-induced dityrosine formation induced by the endogenous release of peroxynitrite during reperfusion of the ischemic heart (28). The antioxidant properties of NO or NO-generating drugs and their ability to reduce the detrimental actions of authentic peroxynitrite have also been observed by others in a variety of biological systems (6, 16, 17, 24, 27).

Urate was shown to be a powerful antioxidant molecule and has been proposed to be a natural scavenger for peroxynitrite in biological systems (9, 21). In our present study, urate showed a remarkable inhibitory effect against peroxynitrite-induced dityrosine formation even at the lowest concentration tested (30 μM), whereas other purine derivatives, such as adenosine, hypoxanthine, and xanthine, had negligible effects even at 1,000 μM. This may support the hypothesis that urate, which has a plasma concentration of 200–400 μM, close to its solubility limit in humans (9), might be a unique and efficacious scavenger of peroxynitrite *in vivo*.

Our results show that the inhibitory profile of the various compounds showed different patterns when different concentrations of peroxynitrite were used to initiate dityrosine formation. This suggests that the inhibitory effect of peroxynitrite scavengers is dependent on the ratio of concentrations of drug:peroxynitrite. Direct interpolation of the present *in vitro* results to *in vivo* conditions should be treated cautiously, because these compounds may have different accessibility to various cellular compartments and different metabolic pathways *in vivo*. This becomes especially relevant considering that peroxynitrite may be generated in mitochondrial, cytosolic, and plasma membrane compartments, sites of both NO and superoxide generation.

This study demonstrates a simple, sensitive, and rapid screening system, using a physiologically relevant buffer system and concentration of peroxynitrite, to test compounds for their peroxynitrite-scavenging properties. It suggests that reduced or oxidized thiols, NO donors, and urate, but not other purine derivatives, are effective scavengers of peroxynitrite in bicarbonate/CO_2-containing buffer *in vitro*.

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ABBREVIATIONS

GSH, reduced glutathione; GSNO, *S*-nitrosoglutathione; GSSG, oxidized glutathione; NO, nitric oxide.

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