TIAPROFENIC ACID-INDUCED PHOTOHEMOLYSIS *IN VITRO* IS INHIBITED BY NIMESULIDE

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SUMMARY

The effect of nimesulide on red blood cell (RBC) lysis photosensitized by tiaprofenic acid was investigated. The tiaprofenic acid-induced photohemolysis rate was enhanced by exposure to oxygen but lysis was also observed under anaerobic conditions. Photohemolysis was decreased by reduced glutathione (GSH) and reduced even more by butylated hydroxyanisole (BHA); sodium azide, superoxide dismutase and mannitol did not show a significant effect. Nimesulide did not cause any RBC lysis and inhibited this action of tiaprofenic acid by 20-30%, depending on the concentration of nimesulide and the intensity of ultraviolet A light. The protective effect of GSH, but not of BHA, was increased by nimesulide. Our findings suggest that free radicals are generated in this *in vitro* model of phototoxicity and are involved in the photoaggression to the red blood cell membrane, this effect being partially inhibited by nimesulide.

KEY WORDS

photosensitization reactions, nimesulide, tiaprofenic acid, photohemolysis, radical scavenger

INTRODUCTION

Phototoxic or photoallergic cutaneous reactions to systemically administered drugs are frequently reported adverse effects. In consequence, *in vitro* models have been developed for screening drugs for these side effects. These models are based on the assumption that the interaction between ultraviolet light and the drug *in vitro* produces events that manifest themselves in a manner similar to the reactions *in vivo*, when cutaneous cells containing the drug are exposed to light.

Since biological membranes have been identified as good experimental targets for the photodynamic action of various sensitizing compounds, studies have been carried out on whole cell preparations (e.g. red blood cells) or cell cultures, in microorganism systems, in isolated cell membranes and subcellular organelles /1,2/. Some of these models, such as the red blood cells, are also useful for the investigation of mechanisms of phototoxicity, including oxygen dependency studies and investigations of the effect of radical scavengers.

In addition to the extensive therapeutic importance of the non-steroidal anti-inflammatory drugs (NSAIDs) as analgesic and antipyretic agents, several members of this group of drugs, such as benoxaprofen /3,4/, carprofen /5,6/, naproxen /7,8/, tiaprofenic acid /9,10/ and other propionic acid derivatives /11/, have been shown to cause photosensitization reactions. One of the mechanisms involved in these reactions could be the production of free radicals, since previous studies have shown that free radicals are produced during photolysis of benoxaprofen /12,13/, naproxen /14/, ketoprofen /15/ and tiaprofenic acid /16/.

Nimesulide has shown special pharmacological characteristics /17/ in that it is more potent than indomethacin or aspirin in inhibiting carrageenin rat paw edema /18/ and ultraviolet-induced erythema of guinea-pig skin /18/. However, this NSAID only exhibited intermediate potency in inhibiting prostaglandin synthesis /19,20/, not affecting the concentration of cytoprotective prostaglandins in the gastric mucosa /21/. There is some evidence to suggest that nimesulide has an oxygen and other free radical scavenger effect /22/ which, theoretically, could inhibit *in vitro* the photosensitization action of these propionic acid derivatives.

This paper describes our studies on the influence of nimesulide on tiaprofenic acid-induced photohemolysis in human erythrocytes, as well as the interaction with known radical scavengers.

MATERIALS AND METHODS

Photohemolysis

Blood (10 ml) was collected by venous puncture from 11 normal human volunteers not taking any drugs. Red blood cells (RBC) of a single donor were prepared by washing three times with a tenfold volume of physiological saline solution, each time centrifuging the cells at 2500 *g* for 15 min and carefully removing the supernatant. The RBC were then diluted in potassium-free Krebs-Henseleit solution containing tiaprofenic acid (100 μmol/l) and/or nimesulide (5-500 μmol/l) so that the resultant suspension had a hematocrit of 2.5%. The drug solutions were bubbled with either oxygen or nitrogen for 20 min prior to the addition of RBC. When required, butylated hydroxyanisole (BHA) (final concentration: 0.01 mmol/l), reduced glutathione (GSH) (1 mmol/l), sodium azide (AZI) (1 mmol/l), superoxide dismutase (SOD) (200 IU/ml) or mannitol (MAN) (10 mmol/l) were added to the drug solutions, before bubbling. These additives were dissolved in potassium-free Krebs-Henseleit solution before addition to the cells. BHA was dissolved previously in methanol. The same concentration of methanol (1%) was added to the control sample.

Finally, the test tubes were tightly sealed and irradiated. Dark controls were run in all experiments and showed no hemolysis.

Irradiation was performed by a PUVA unit (Psoralite, Paul B Elder Company, Ohio, USA) equipped with 44 lamps (Voltarc, USA, F72T12-BL-HO) having an emission peak at 365 nm and an output of 16.0 mW/cm^2 at a distance of 15 cm as measured with a UVA meter (VLX-365, Vilber Lourmat, France). A merry-go-round irradiation apparatus was used to ensure that all samples received equal radiation. The reaction cells were thin wall nuclear magnetic resonance (NMR) tubes (ICN Biomedicals, Inc, USA) of 5 mm diameter, and a surface of irradiation of 8 cm^2 per 1 ml of sample volume.

After irradiation, the RBC suspension was centrifuged and the hemolysis rate was determined by measuring the potassium concentration by flame photometry in the supernatant (Jencons Scientific Ltd., England).

Analysis of data and statistics

Each type of experiment was performed at least five times (n=5), each time in triplicate and with the blood of a different donor. The mean of the triplicate was used for analysis.

Calculations of hemolysis (determined by potassium in the supernatant) are presented as a percentage of complete hemolysis obtained by hypotonic shock.

Results are expressed as the means ± SEM. Means were analyzed for statistical differences using one-way analysis of variance (ANOVA) and Student's t-test. $P \leq 0.05$ was considered significant.

Drugs and reagents

Tiaprofenic acid and nimesulide were gifts from Roussell (Portugal) and Helsinn (Portugal), respectively.

Butylated hydroxyanisole (BHA), reduced glutathione (GSH), sodium azide (AZI), superoxide dismutase (SOD) and mannitol (MAN) were obtained from Sigma Chemical Company. All other chemicals were reagent grade.

RESULTS

Tiaprofenic acid and nimesulide did not cause any lysis of erythrocytes kept in the dark. However, when irradiated by UVA light (Fig. 1 - control) tiaprofenic acid caused photohemolysis, which was intensified when the solution was bubbled with oxygen (Fig. 2). Bubbling with air induced a lesser effect (Fig. 2). In the absence of oxygen (solution gassed with nitrogen), the photohemolysis rate was the lowest (Fig. 2). Nimesulide did not induce any photohemolysis.

When nimesulide was added to the tiaprofenic acid solution, the rate of photohemolysis was significantly decreased (p=0.01 by ANOVA) in a concentration dependent way (Fig. 1), principally at low UVA intensity and in solutions previously bubbled with oxygen (Fig. 2). In the solution gassed with nitrogen, nimesulide did not give any protection against hemolysis (Fig. 2). Of the three concentrations of nimesulide used (Fig. 1), only 50 and 500 μmol/l decreased hemolysis significantly. We chose a concentration of 100

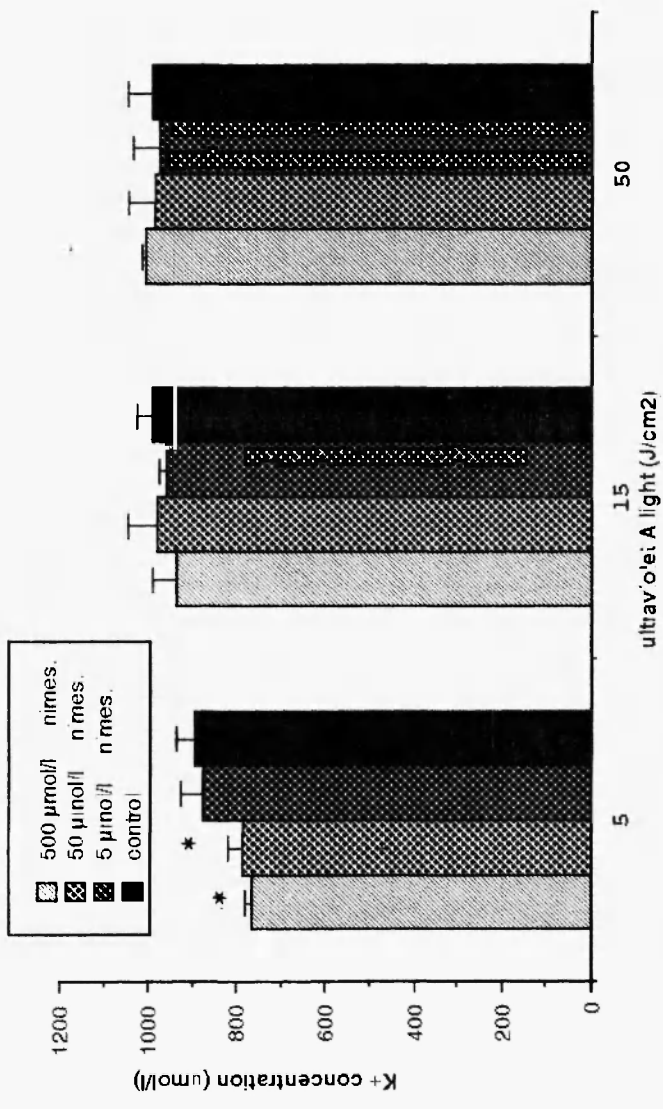

Fig. 1: Decrease by various concentrations of nimesulide of the UVA-induced lysis of red blood cells sensitized by 10⁻⁵ µmol/l tiaprofenic acid. The experiments were performed under exposure to air. The hematocrit was 2.5%. Control: the UVA-induced lysis of erythrocytes sensitized by tiaprofenic acid but without nimesulide. *P<0.05 (ANOVA) compared to control. The results represent means ± SEM (n=5).

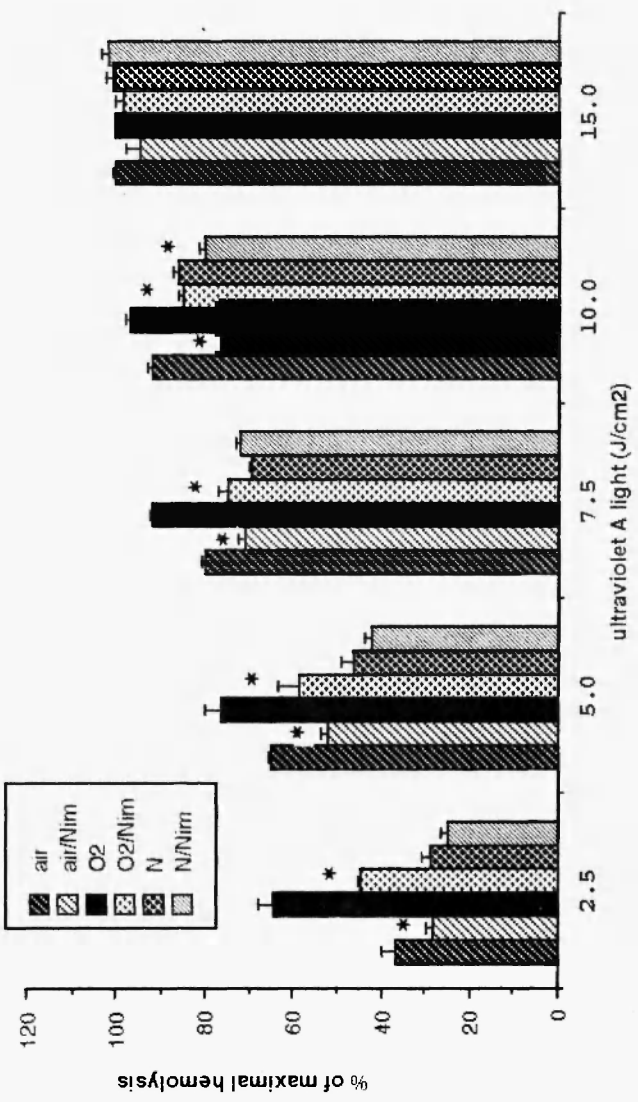

Fig. 2: Decrease of UVA-induced lysis of erythrocytes sensitized by 100 μmol/l tiaprofenic acid. Before the addition of the red blood cells (final hematocrit of 2.5%), the solutions with or without 100 μmol/l nimesulide were bubbled with air, oxygen or nitrogen (N). *P<0.05 (Student's t-test) for difference between hemolysis in the absence or presence of nimesulide. The results represent means ± SEM (n=5).

μmol/l for the following experiments.

BHA significantly decreased the tiaprofenic acid-induced photohemolysis (Fig. 3). GSH also diminished this effect but to a lesser extent (Fig. 4). When nimesulide was added to these treated suspensions of RBC, the photohemolysis was only decreased in the GSH treated solutions (Fig. 4). The tiaprofenic acid-induced photohemolysis was not affected by sodium azide, superoxide dismutase or mannitol (Fig. 5).

DISCUSSION

Photosensitized hemolysis has been attributed to membrane damage which results in disturbed cation permeability and destruction of the osmotic equilibrium of the intact cell. As a consequence potassium leaks through the membrane and its measurement in the extracellular fluid reflects the erythrocyte damage. This technique is more sensitive and gave us more reproducible results than determination of the hemoglobin content in the supernatant /16/.

This study corroborated our observation /16/ that low concentrations of tiaprofenic acid can induce photohemolysis of human erythrocytes. Similar results for benoxaprofen, naproxen and ketoprofen were reported by Ferguson *et al.* /23/ and Costanzo *et al.* /14,15/. However, there is some controversy about the oxygen dependence of this effect. Webster *et al.* /24/ demonstrated *in vitro* that in the presence of UV radiation benoxaprofen produced a dose-dependent lysis of sheep erythrocytes that did not require the presence of oxygen. Photohemolysis induced by naproxen /14/ and ketoprofen /15/ also occurs under anaerobic conditions but the presence of oxygen markedly enhances the cell lysis. In the present study we found that tiaprofenic acid, like other propionic acid NSAID derivatives, induces photohemolysis under aerobic and anaerobic conditions, but this effect is more evident in the presence of oxygen. These observations suggest that tiaprofenic acid can cause membrane damage by both oxygen-dependent and independent mechanisms.

The protection provided by nimesulide could be due to a radical scavenger effect. With the aim of providing further information on the mechanism of action, the photohemolysis studies were repeated in the presence of two free radical scavengers, BHA and GSH; of a superoxide (O_2^-) scavenger, SOD; of a singlet oxygen quencher,

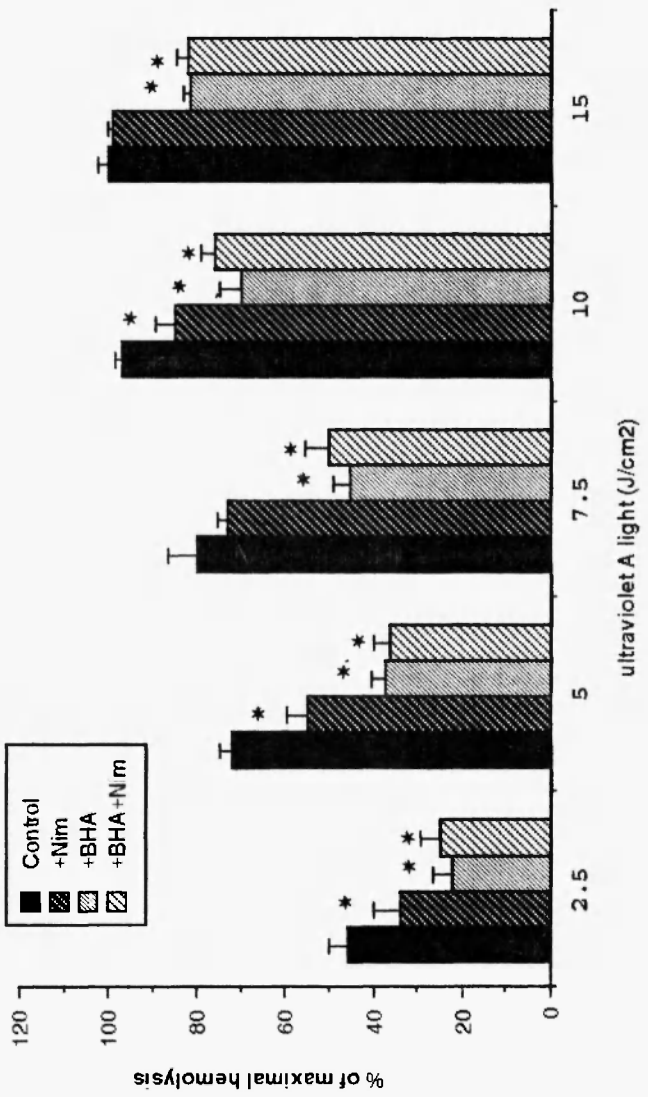

Fig. 3: Protection by 10 μmol/l butylated hydroxyanisole (BHA) and/or 100 μmol/l nimesulide of UVA-induced lysis of erythrocytes sensitized by 100 μmol/l tiaprofenic acid. Before adding the erythrocytes, the drug solutions were bubbled with oxygen. *P<0.05 (Student's t-test) compared to control (UVA-induced hemolysis in presence of only tiaprofenic acid). The results represent means ± SEM (n=3).

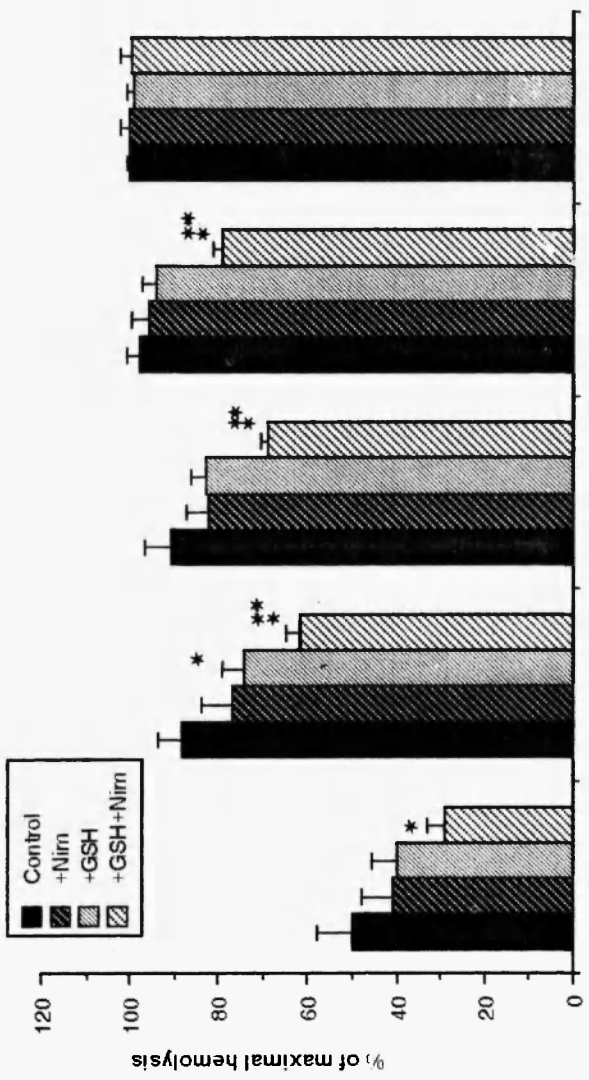

Fig. 4: Protection by 1 mmol/l reduced glutathione (GSH) and/or 100 μmol/l nimesulide of UVA-induced lysis of erythrocytes sensitized by 100 μmol/l tiaprofenic acid. Before adding the erythrocytes, the drug solutions were bubbled with oxygen. *P<0.05 (Student's t-test) compared to control (UVA-induced hemolysis in presence of only tiaprofenic acid). **P<0.05 compared to samples treated with nimesulide or GSH. The results represent means ± SEM (n=5).

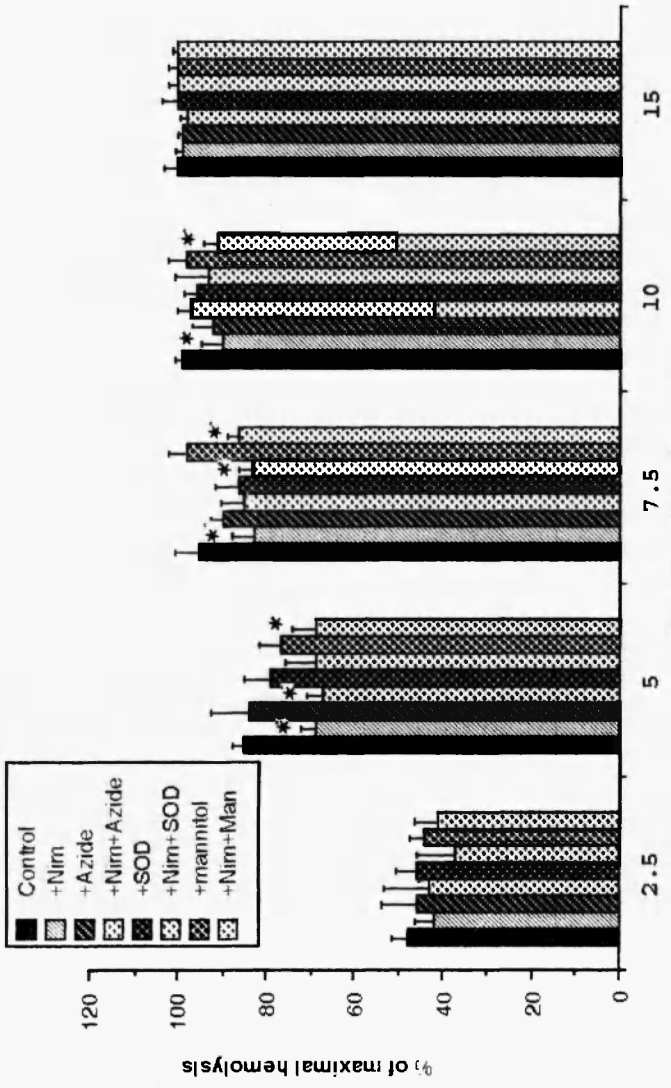

Fig. 5: Protection by 1 mmol/l sodium azide, 200 IU/l superoxide dismutase (SOD) or 10 mmol/l mannitol, and/or 100 μmol/l nimesulide, of UVA-induced lysis of erythrocytes sensitized by 100 μmol/l tiaprofenic acid. Before adding the erythrocytes, the drug solutions were bubbled with oxygen. *P<0.05 (Student's t-test) compared to control (UVA-induced hemolysis in presence of only tiaprofenic acid). The results represent means ± SEM (n=5).

AZI; and of a hydroxyl radical scavenger, MAN. In fact, the ability of BHA and GSH to provide partial protection against tiaprofenic-acid induced photohemolysis strongly suggests that free radicals are involved in the membrane damage; the lower capacity of reduced glutathione, a water-soluble radical scavenger, to protect against this photohemolytic effect is probably due to the inability of this large and polar molecule to penetrate easily into the membrane. The observation that sodium azide does not protect against tiaprofenic acid-induced lysis seems to rule out the involvement of singlet oxygen in the process. However, it should be pointed out that the erythrocyte membrane itself has a scavenger effect upon singlet oxygen /25/ and that sodium azide is able to inhibit endogenous catalase and superoxide dismutase activities /26/, rendering the red blood cell membrane more sensitive to radical injury. Similarly, the lack of efficacy of superoxide dismutase does not mean that superoxide is not generated in this "biological" model of phototoxicity, since the erythrocyte levels of catalase, glucose-6-phosphate dehydrogenase, glutathione peroxidase and superoxide dismutase may protect these cells against some of the oxidative photoaggression.

Costanzo *et al.* /14/ demonstrated that SOD and AZI inhibit naproxen-induced photohemolysis, but they also showed that the red blood cell photolysis sensitized by ketoprofen is unaffected by sodium azide /15/.

In the tiaprofenic acid-induced photohemolysis, out of the substances studied here, nimesulide caused an additional reduction only with GSH. BHA protection was not modified and the other radical scavengers did not affect the reduction by nimesulide. These findings suggest that nimesulide may have a radical scavenger effect, and we can hypothesize that it has a similar mechanism to that of BHA. The action of GSH was additive to that of nimesulide.

In conclusion, we demonstrated that tiaprofenic acid induces hemolysis in UVA light, an effect enhanced by exposure to oxygen. Nimesulide, a non-steroidal anti-inflammatory drug with weak potency in the inhibition of cyclooxygenase, decreased the tiaprofenic acid-induced photohemolysis, principally when the drug solutions were bubbled with oxygen. A similar capacity was demonstrated for BHA. GSH showed a synergistic action with nimesulide. Therefore, it seems possible to conclude that, in this model, nimesulide acts as a radical scavenger.

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