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ORIGINAL ARTICLE

Pharmacokinetics and tissue and tumor exposure of CP-31398, a p53-stabilizing agent, in rats

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Abstract
Purpose CP-31398 (N′-[2-[(E)-2-(4-methoxyphenyl)ethenyl]quinazolin-4-yl]-N,N-dimethylpropane-1,3-diamine hydrochloride) is one of the new class of agents that can stabilize the DNA-binding domain of p53 and thereby maintain the activity of p53 as a tumor suppressor and transcription factor. Through its activity as a p53 stabilizer, CP-31398 demonstrates significant cancer preventive and therapeutic activity in several in vivo animal models. The objective of the current study was to describe the pharmacokinetic profile and tissue distribution of this novel agent following intravenous or oral (gavage and dietary) administration.
Methods CP-31398 was administered to male CD and F344 rats as a single intravenous bolus dose or by daily oral gavage dosing. Male F344 rats also received drug as an ad libitum dietary supplement. Plasma, liver, skin, colon, and colon tumor samples were collected after oral dosing. Concentrations of CP-31398 in plasma and tissue samples were analyzed using LC–MS/MS, and the resultant data were subjected to a non-compartmental pharmacokinetic analysis.
Results Bioavailability (12–32%), elimination half-life (14–20 h), clearance (4.2–4.8 l/h/kg), and volume of distribution (70–82 l/kg) were determined. Tissue levels of CP-31398 after oral (gavage or diet) administration were several orders of magnitude higher than were corresponding plasma concentrations; CP-31398 levels were especially high in colon and liver. Levels of CP-31398 in tissues were higher after gavage dosing than after dietary administration.
Conclusions CP-31398 is bioavailable and has a relatively long elimination half-life, which supports the achievement of plasma steady-state levels with a once daily dosing regimen. CP-31398 exhibits a dramatically high volume of distribution, which is consistent with its tissue concentrations being much higher than corresponding plasma levels. It is accumulated in colon tumor tissues, albeit at lower concentrations than found in liver, skin, and colon.

Keywords p53 · Chemoprevention · Pharmacokinetics · Liver · Colon tumor · Rat

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Introduction

The tumor suppressor protein p53 is a transcription factor that plays an important role in cell cycle arrest and apoptosis [1, 6, 13, 16]. Various cellular stressors, for example, DNA damage, oncogene activation, and hypoxia, induce p53 expression [8]. Because more than 50% of human cancers demonstrate reduced or lost function of the p53 tumor suppressor gene [17], restoration or stabilization of

p53 function through administration of small molecules presents an attractive strategy for cancer prevention and therapy [3, 5, 6, 17–19].

The synthetic styrylquinazoline, CP-31398 (N′-[2-[(E)-2-(4-methoxyphenyl)ethenyl]-quinazolin-4-yl]-N,N-dimethylpropane-1,3-diamine hydrochloride)] [12], was first identified in a high-throughput screening system for its ability to restore the wild-type conformation to DNA-binding domain of p53 [7]. It stabilizes the wild-type DNA-binding conformation of the p53 protein and can restore the DNA-binding activity of mutant p53 [15, 17, 19]. Presumably as a result of its activity in the stabilization of p53 and/or the restoration of the wild-type phenotype to cells in which p53 has been mutated, CP-31398 demonstrates a range of cancer chemopreventive activities in animal models. In the colon, CP-31398 protects against cancer induction in APC ($min^{+/-}$) mice [12] and inhibits cancer induction in rats by the chemical carcinogen, azoxymethane [11]. CP-31398 also inhibits the induction of skin cancers in mice by UVB radiation [14]. On the basis of its demonstrated cancer preventive activity in rodent models, CP-31398 came under intensive study as a proof-of-principle agent and entered a preclinical drug development evaluation path.

This report presents data characterizing plasma, tissue (colonic mucosa, skin, and liver), and colonic tumor levels of parent drug following oral (gavage or dietary supplementation) or intravenous administration of CP-31398 to CD and F344 rats. Both routes of oral administration of CP-31398 represent approaches used in the preclinical development of this agent: GLP-compliant preclinical toxicology studies of CP-31398 in rats have been performed using oral (gavage) administration of the drug [9], while chemoprevention efficacy evaluations in rodent models have been performed using dietary supplementation with this agent [11, 12]. Similarly, preclinical toxicology studies were performed in CD rats, while colon cancer chemoprevention studies were performed using an experimental model that was developed in F-344 rats. Thus, pharmacokinetic evaluations were performed in both rat strains.

Experimental methods

Animal welfare considerations

Prior to the initiation of in vivo experimentation, study protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the performing organizations. All aspects of the program involving animal care, use, and welfare were performed in compliance with United States Department of Agriculture regulations and the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and by the US Department of Agriculture through the Animal Welfare Act (7 USC 2131, 1985) and Animal Welfare Standards incorporated in Title 9, Part 3 of the *Code of Federal Regulations*, 1991.

Test article

CP-31398 (>99% purity; Indofine Chemical Co., Hillsborough, NJ) was protected from light and stored desiccated at 2–8°C under nitrogen. Gavage dosing formulations were prepared by dissolving CP-31398 in ASTM Type 1 water; after preparation, dosing formulations were stored under nitrogen at 2–8°C until used. CP-31398 was protected from light during all dose preparation, storage, and dose administration operations. Rats received daily oral (gavage) exposure to CP-31398 or vehicle at doses of 40 or 80 mg/kg/day for 28 consecutive days.

Dietary admixtures were prepared by mixing CP-31398 with AIN-76, a semi-purified, casein-based diet. CP-31398 was administered at a level of 150 and 300 mg/kg diet for 31 weeks. Diets were based on the modified AIN-76A diet. The semi-purified diet includes 20% casein, 52% corn starch, 13% dextrose, 5% corn oil, 5% alphacel, 3.5% AIN mineral mix, 1.2% AIN revised vitamin mix, 0.3% D,L-methionine, and 0.2 choline bitartrate (26).

CP-31398 was premixed with a small quantity of diet and then blended into bulk diet using a Hobart mixer. Both control and experimental diets were prepared weekly and stored in a cold room.

Animals

Male rats were used throughout. CD (Crl:CD®[SD]IGS) rats of about 7 weeks of age (terminal weight ~330 g) were used in the gavage and intravenous administration studies. F-344 (F344/NCrl) rats that had been treated with the carcinogen azoxymethane (AOM, 15 mg kg BW, s.c., once weekly for 2 weeks at age of 8 and 9 weeks, terminal weight ~470 g) were used in the dietary administration studies. Dietary doses were determined based on the maximal tolerated dose (MTD) in the animal chemopreventive studies [11]. MTD was defined as the highest dose that causes no more than a 10% body weight decrement or produces mortality or any external signs of toxicity that would be predicted to shorten the natural life span of the animal. Gavage doses were selected in rats on the basis of the results in a preliminary 14-day range-finding toxicology study. For comparison, gavage equivalent daily doses (in mg/kg bw/day) were estimated from the dietary doses (in ppm) after taking into account animal average weight and their average daily food consumption (as in Table 3).

Analytical methods

Plasma

For the determination of CP-31398 in plasma, a 100 µl plasma aliquot was mixed with 1 ml of acetonitrile. After vortex mixing for 1 min, the sample was centrifuged at 4°C and 7,000 RPM for 10 min to remove precipitated proteins, and the supernatant was transferred to a clean tube and dried under nitrogen flow at room temperature (approximately 25°C). After the evaporation was completed, the residue was reconstituted in 500 µl of methanol/water (v/v 20:80) and vortex-mixed and centrifuged again. An aliquot of the resulting supernatant was transferred to an autosampler vial for instrumental analysis.

Tissue

Colons were rinsed with normal saline (to remove the fecal content) and cut open; colonic mucosa was scrapped with a microscopic slide at 4°C (on ice). Standard techniques were employed in sampling other tissues. Tissue specimens were stored frozen at −70°C until processing. For the determination of CP-31398 in tissue, specimens were thawed, weighed (weight range for specimens was 5–44, 120–400, and 300–700 mg for tumor, mucosa, and liver tissue, respectively), and homogenized in 0.9% sodium chloride injection USP at ratios of 1:2 (w/v) for mucosa and liver or in 0.05 ml for tumor, respectively. The tissue homogenates were stored frozen at −70°C until analysis. A 50 or 100 µl tissue homogenate aliquot was mixed with 0.5 ml of 1% formic acid in water (1% FA solution) and vortex-mixed and sonicated for 7 min. After the addition of 100 µl of ammonium hydroxide, the sample was extracted twice with 0.8 ml of methyl tert-butyl ether and centrifuged, and the supernatant was transferred to a clean tube and dried under nitrogen flow. After the evaporation was completed, the residue was reconstituted in 500 µl of methanol/1% FA solution (v/v 20:80) and vortex-mixed and centrifuged again. An aliquot of the resulting supernatant was transferred to an autosampler vial for instrumental analysis.

Plasma and tissue samples were analyzed for concentration of CP-31398 using liquid chromatography–tandem mass spectrometry (LC–MS/MS) [10]. Levels of CP-31398 in tissue were measured using a tandem mass spectrometer (API 3000; Applied Biosystems/MDS Sciex, Foster City, CA) equipped with a high-performance liquid chromatograph (Agilent 1100; Agilent Technologies, Wilmington, DE). The chromatographic column was a Luna 3 µm phenyl-hexyl 30 × 2.0 mm (Phenomenex, Torrance, CA). The column temperature was maintained at 25°C, and a flow rate of 0.30 ml/min was used. The mobile phase (MP) consisted of MP-A, 1% formic acid in water, and MP-B, 1% formic acid in methanol. The mobile-phase gradient was as follows: after injection, initial conditions with MPA at 65% were held for 0.5 min, decreased to 5% in 0.5 min and held constant for 3 min, returning to initial conditions for another 3 min of re-equilibration time. Retention time for the CP-31398 was 1.5 min. Total run time was 7 min. A turbo ion spray interface was used as the ion source operating in positive ion mode. Acquisition was performed in multiple reaction monitoring mode using the following ions: 363.4 (Q1) and 318.2 (Q3). Ion spray voltage was 4,500 V, ion source temperature was 450°C, and collision energy was 10 V.

Pharmacokinetic analysis

Mean (±SD) plasma concentration–time profiles were calculated from four replicate plasma concentration measurements per time point. Mean plasma concentration–time profiles of CP-31398 in the rats at scheduled (nominal) sampling times were analyzed by non-compartmental pharmacokinetic methods using WinNonlin® Professional Edition software, Version 5.0.1 (Pharsight Corporation, Mountain View, CA). Key parameters, including T_{max}, C_{max}, AUC_{0-t}, AUC_{0-inf}, and $t_{½}$, were calculated for both routes of administration. $t_{½}$, was calculated with non-compartmental analysis using the formula:

$$t_{1/2} = \ln 2/\text{lambda } z \text{ or } 0.693/\lambda z$$

Three points were used to define the terminal phase: 12, 24, and 48 h. Using these three points, a terminal rate constant (λz) was determined to be 0.0485 and 0.0351 for F-344 and CD rats, respectively, and $r^2 > 0.998$.

Additional parameters were calculated as appropriate: $\%F$ was calculated for the oral route, and C_0, CL, and V_{ss}, for the intravenous route. Bioavailability was calculated according to the following formula:

$$\%F = \left\{\left(AUC_{po,tau} \times \text{Dose}_{iv}\right)/\left(AUC_{iv,0-inf} \times \text{Dose}_{po}\right)\right\} \times 100$$

where tau is the dosing interval (24 h) on day 28 and $AUC_{iv,0-inf}$ was obtained on day 1.

Results

In order to determine the bioavailability of CP-31398, the pharmacokinetic profile of this agent was determined in both CD and F344 rats receiving a single intravenous injection of CP-31398 at a dose of 5 mg/kg body weight. The plasma concentration–time profile for each rat strain is shown in Fig. 1; pharmacokinetic parameters for intravenous administration of CP-31398 are summarized in Table 1.

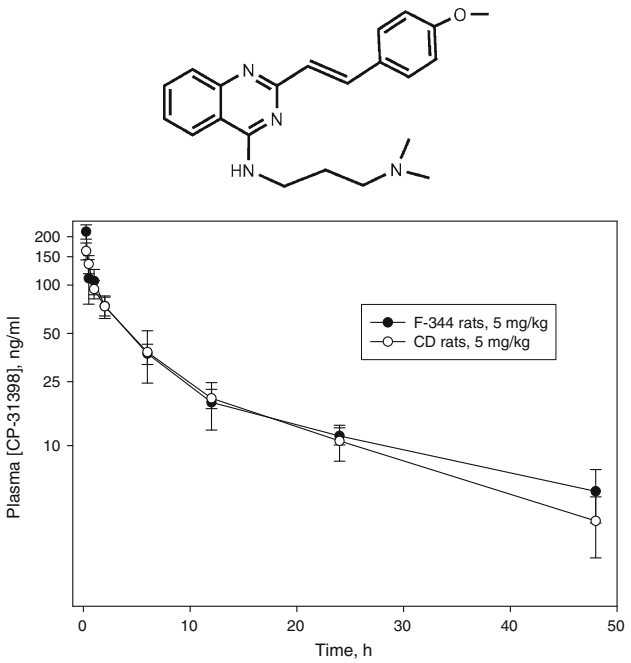

Fig. 1 Plasma concentration–time profile after a single bolus intravenous administration of CP-31398 to rats

Table 1 Pharmacokinetic parameters following a single intravenous dose in male F344 and CD rats

Strain	Dose (mg/kg)	C_0 (ng/ml)	$AUC_{0-\infty}$ (h ng/ml)	$t_{½}$ (h)	CL (l/h/kg)	V_{ss} (l/kg)
F344	5	420	1,184	19.8	4.2	81.8
CD	5	197	1,043	14.3	4.8	69.7

Overall, clearance curves for CP-31398 in CD rats and F344 rats were quite similar following intravenous administration of the drug. Plasma levels of CP-31398 in the two strains were comparable at all time points during the first 24 h after administration, and plasma concentration curves in the two rat strains were essentially superimposable throughout this period. Clearance of CP-31398 from the plasma appeared to be somewhat greater in CD rats than in F344 rats between 24 and 48 h after dosing; however, the difference in plasma drug levels in the two strains at 48 h after administration was not statistically significant. C_0, $t_{1/2}$, CL, and V_{ss} were 420 ng/ml, 19.8 h, 4.2 l/h/kg, and 81.8 l/kg, respectively, in F-344 rats, and 197 ng/ml, 14.3 h, 4.8 l/h/kg, and 69.7 l/kg, respectively, in CD rats.

Plasma drug level data for the two rat strains after daily gavage exposure to CP-31398 doses of 40 or 80 mg/kg/day for 28 consecutive days are illustrated in Fig. 2. Plasma concentration–time profiles generated after the last dose suggest essentially a steady state in both strains after both dose levels. Plasma drug levels were related to administered dose, and plasma drug levels in both dose groups

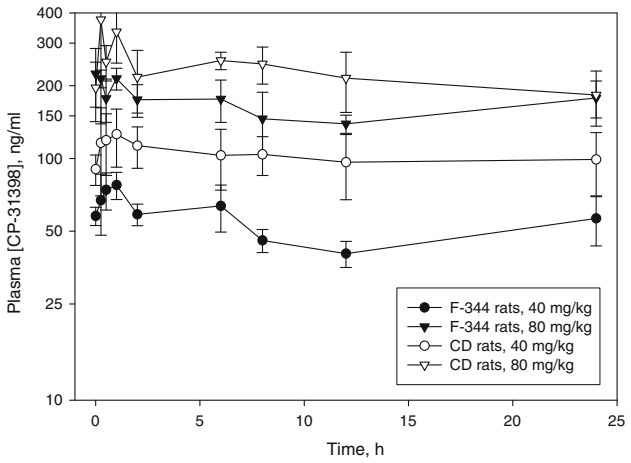

Fig. 2 Plasma concentration–time profile after daily oral gavage dosing of CP-31398 to rats

were consistently greater in CD rats than in F-344 rats. Pharmacokinetic parameters for CP-31398 after 28 days of gavage exposure, including bioavailability, are summarized in Table 2. Oral bioavailability after a 28-day exposure to CP-31398 at doses of 40 and 80 mg/kg/day, respectively, were 13.2 and 12.4% in F-344 rats and 29.3 and 31.9% in CD rats.

Levels of CP-31398 in plasma and in target tissues in which CP-31398 has demonstrated chemopreventive activity (liver and skin) are presented in Table 3 for F-344 rats receiving gavage exposure or dietary supplementation

Table 2 Pharmacokinetic parameters following 28-day oral gavage dosing in male F344 and CD rats

Strain	Dose (mg/kg)	T_{max} (h)	C_{max} (ng/ml)	AUC_{0-24} (h ng/ml)	*%F*
F344	40	1.0	77.6	1,247	13.2
F344	80	1.0	128	2,352	12.4
CD	40	1.0	126	2,449	29.3
CD	80	0.25	375	5,323	31.9

Table 3 Plasma and tissue levels of CP-31398 following oral gavage and diet dosing in male F344 rats obtained 22–26 h after the last dose

	80 mg/kg bw/day po gavage	40 mg/kg bw/day po gavage	~29 mg/kg bw/day 375 ppm diet
Plasma (ng/ml)	73.6 (26)	35.7 (5.6)	24.7 (4.3)
Liver (μg/g)	3,250 (780)	540 (220)	118 (21)
Skin (μg/g)	117 (71)	19.6 (6.9)	5.6 (1.6)
Liver/plasma	44,157	15,126	4,777
Skin/plasma	1,590	549	227

Plasma and tissue concentrations are presented as mean (±SD)

Table 4 Plasma and tissue levels of CP-31398 following dietary dosing in cancer chemopreventive model in F-344 male rats

	150 ppm diet	300 ppm diet
Plasma (ng/ml)	8.7 (2.2)	20.8 (9.8)
Liver (μg/g)	13.7 (4.6)	113 (83)
Colonic mucosa (μg/g)	3.3 (1.7)	13.3 (7.3)
Colon tumor (μg/g)	0.91 (0.32)	4.38 (2.2)
Liver/plasma	1,584	5,413
Colonic mucosa/plasma	381	641
Colonic tumor/plasma	105	210

Plasma and tissue concentrations are presented as mean (±SD)

with the drug. Plasma levels of CP-31398 levels were proportional to dose and were in the ng/ml range at both gavage doses and the single dietary level studied. Levels of CP-31398 in the liver and skin were also proportional to dose, but demonstrated a much steeper dose–concentration relationship. Perhaps more important, tissue levels of CP-31398 were in the μg/g range. Concentrations of CP-31398 levels were especially high in the liver, an identified target tissue for the toxicity of this agent in rats [9], resulting in very high tissue-to-plasma concentration ratios.

Plasma levels of CP-31398 measured in rats receiving gavage exposure at 40 mg/kg were roughly comparable to those in the dietary exposure group that received approximately 29 mg CP-31398/kg bw/day. However, although plasma levels in these groups differed by less than a factor of two (35.7 ng/ml vs. 24.7 ng/ml), drug levels in the liver and skin of rats receiving gavage exposure to CP-31398 at 40 mg/kg/day were three- to fivefold higher than were levels in those tissues following dietary exposure.

Plasma and tissue concentrations of CP-31398 after dietary exposure of carcinogen-treated F344 rats at levels of 150 and 300 mg/kg diet for 31 weeks are summarized in Table 4. Plasma and liver concentrations were generally comparable to those in non-carcinogen-treated F-344 rats following 375 ppm dietary administration of CP31398 (Table 3). While plasma concentrations of CP-31398 were roughly proportional to the dose, tissue concentrations increased disproportionally to the dose. Liver and colon concentrations of CP-31398 again greatly exceeded those of plasma. CP-31398 also preferentially accumulated in colonic tumors relative to plasma but to a lesser extent than in colon or liver.

Discussion

Loss or reduced p53 function is the most common alteration in human cancers [17]; on this basis, maintenance or restoration of wild-type p53 activity presents an attractive target for the design of drugs for cancer prevention and therapy [4]. CP-31398 belongs to an emerging class of compounds with a potential of restoring and/or stabilizing the functional activity of p53 [2]. Evaluations of its pharmacokinetic profile and tissue levels (including tumors) were integrated into preclinical toxicology and efficacy studies in CD and F344 rats after oral gavage and oral diet dosing. Oral (gavage) dosing in CD rats is the standard approach used in preclinical toxicology studies, while dietary dosing in F344 rats is used in colon cancer chemoprevention studies. Intravenous dosing was included in order to enable determinations of CP-31398 bioavailability. Its concentration in target organs, colon and skin, was determined as well as in liver, the organ which experiences the major presystemic exposure of orally administered drugs.

CP-31398 exhibits a relatively long elimination half-life of 14–20 h. As a result of its long half-life, a steady-state plasma drug level was achieved with once per day bolus oral dosing. Clearance of CP-31398 from the plasma was slightly higher than the hepatic blood flow (4 l/h/kg) in rats. However, the most surprising parameter was the vast volume of distribution of CP-31398, V_{ss}, was equal to or greater than 70 l/kg. This volume of distribution is two orders of magnitude greater than is the total body water in rats ($\sim$0.7 l/kg) and suggests very extensive tissue distribution of this drug.

Consistent with this observation, tissue (liver, skin, and colon) concentrations of CP-31398 following oral exposure were several orders of magnitude greater than were plasma concentrations in the same exposure groups. Furthermore, while plasma concentrations appeared to increase generally in proportion to dose, tissue concentrations of CP-31398 demonstrated a much greater increase with dose. The pattern of very extensive tissue accumulation of CP-31398 relative to its plasma concentration was evident in both rat strains and after both oral gavage and dietary dosing.

The extensive hepatic deposition of CP-31398 appears likely to underlie the sensitivity of the liver to the toxicity of this agent. In a 28-day oral toxicity study in rats, the liver was found to be the primary site of limiting toxicity for CP-31398 [9]: several rats exposed to the high dose (160 mg/kg/day by gavage) of CP-31398 used in this study died of massive hepatic coagulation necrosis (hepatic infarcts). Although no mortality was observed in rats receiving drug via gavage at 80 or 40 mg/kg/day, several rats exposed to CP-31398 at 80 mg/kg/day demonstrated hepatic infarcts that were less severe than those seen in the higher dose group. Furthermore, statistically significant increases in the levels of several serum enzymes (alkaline phosphatase and alanine aminotransferase) that serve as indicators of hepatic damage were seen in animals

receiving drug at 80 mg/kg/day. CP-31398 also accumulated in the colon tumors but to a lesser degree than in non-tumor tissues.

The in vivo metabolic profile of CP-31398 was recently described [9]. The most abundant metabolite is the N-demethylated product, and the other major metabolites include O-demethyl and two hydroxylated products. Presently, it is not clear whether the efficacy and/or toxicity of the parent compound is related to these metabolites. Structural alterations at these sites of metabolism could possibly alleviate some of the toxicity of the parent drug.

CP-31398 was also shown effective in animal skin cancer prevention model following topical application [14]. Topical application could obviate some of the systemic toxicity following its oral administration.

The reasons behind such an extensive CP-31398 accumulation in tissues are presently unclear. CP-31398 was tested for potential interaction with seven drug transporters (OAT1, OAT3, OCT2, OATP1B1, OATP1B3, BCRP, and P-gp) as an inhibitor and/or substrate. In these studies, 30 μM CP-31398 inhibited probe substrate transport of OCT2, P-gp, and BCRP by 52 ± 14, 34 ± 1, and $16 \pm 3\%$, respectively. CP-31398 (10 μM) was not a substrate of the tested drug transporters (unpublished data). Therefore, the compound does not appear to interfere with its own transport mechanisms, at least as far as these seven human transporters were concerned.

The very extensive tissue accumulation of CP-31398 appears to have important biological and toxicological implications. Furthermore, the results of the present study demonstrate that the plasma concentration of CP-31398 does not necessarily serve as a useful indicator of tissue drug concentrations; this factor clearly complicates dosing management of this compound.

In consideration of the extensive hepatic accumulation of CP-31398 that was demonstrated in rats in the present study, and the identification of the liver as the primary site of CP-31398 toxicity in rats, development of drug congeners with less hepatic accumulation may result in a substantive improvement in the ratio of chemopreventive activity to agent toxicity. Several regions in the CP-31398 molecule are suitable for structural manipulation; doing so may yield CP-31398 analogs or congeners that maintain much or all of their p53-stabilizing activity while inducing substantially less toxicity.

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