Reliability of phototoxic tests of fluoroquinolones *in vitro*

ZHANG Ting, LI Jun-Ling, MA Xiao-Chao, XIN Jian, TU Zeng-Hong[1]

State Key Laboratory of Drug Research , Shanghai Institue of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

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ABSTRACT

AIM: To make sure the reliability of phototoxic tests *in vitro* by comparing the phototoxic potential of 4 fluoroquinolones (FQ). **METHODS:** Lomefloxacin (LFLX), sparfloxacin (SPFX), ciprofloxacin (CPFX), and norfloxacin (NFLX) were tested by Wistar rat phototoxic test and Balb/c mouse phototoxic test *in vivo,* and Chinese hamster V79 cell micronucleus test and NIH 3T3 MTT test *in vitro* under the different condition of UVA irradiation. **RESULTS:** In all experiments, LFLX and SPFX showed higher phototoxic potential compared with the control ($P<0.01$ *vs* 1 % CMC), CPFX was mild ($P<0.05$ *vs* physiological saline), NFLX did not show phototoxicity *in vivo*, however at a higher concentration (10 μmol/L) *in vitro*, it also induced phototoxicity as other FQ. **CONCLUSION:** There are good correlations between phototoxic tests *in vivo* and *in vitro.* These results ensure the validation of phototoxic tests *in vitro.*

INTRODUCTION

The fluoroquinolone (FQ) antibiotics were introduced to clinical therapy from the 1980s and are effective against a broad spectrum of bacterial species with low incidence of serious adverse events[1-3]. However, they could photosensitize human skin to solar UV radiation, even inducing skin tumor formation in hairless mice[4,5]. Most of phototoxic reactions are acute and reverse, resolution occurs when FQ are discontinued. In order to avoid phototoxic reaction, patients who are allergic to phototoxicity induced by FQ should avoid extra exposure.

However, the extent of skin phototoxicity of an FQ strongly differs from that of other FQ. For instance, sparfloxacin (SPFX) and lomefloxacin (LFLX) are much more phototoxic drugs than other FQ. The reason why they are different is not clear. From the structure-activity relationship, SPFX and LFLX have the same constituent at the X_8 position responsible for phototoxic potential.

This study was performed to compare the phototoxic potential of 4 marketed FQ *in vivo* and *in vitro* in order to make sure the reliability of phototoxic test *in vitro* and the correlation between *in vivo* and *in vitro* under different UVA irradiation.

MATERIALS AND METHODS

Animals Wistar rats (aged 7-8 weeks and weighing 200 g±20 g), female Balb/c mice (aged 5 weeks and weighing 19 g±2 g, Grade II) were purchased from Shanghai experimental animal center, Chinese Academy of Sciences. They were housed in plastic cages for 1 week for acclimation to the laboratory environment of air-conditioned room (temperature, 25° C±2° C; humidity,

[1] Correspondence to Prof TU Zeng-Hong.
Phn 86-21-6431-1833, ext 325. Fax 86-21-6437-0269
E-mail zhtu@mail.shcnc.ac.cn
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55 %±15 %; lighting cycle, 12 h/d) until use. Commercial laboratory chow (F-2, Shanghai experimental animal center of CAS) and chlorinated tap water were available *ad libitum*. Balb/c mice were albino in the skin and eye. All animals were administered ig as a clinical route of administration for FQ.

Cell culture Chinese hamster lung V79 cells were cultured in RPMI-1640 medium (Gibco-BRL, New York, USA) supplemented with glutamine 2 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 mg/L and 20 % newborn bovine serum. The cells were cultured in plastic T-50 flasks (Nunc, Roskilde, Denmark) at 37 ° C in a humidified atmosphere containing 5 % CO_2. NIH 3T3 cells were cultured in Dulbecco' s modified Eagle' s medium (DMEM); other culture conditions are the same as V79 cells.

Chemicals SPFX was from Beite Pharmaceutical Co, lot number: 2001-04-5. LFLX was from Changzhou Pharmaceutical Co, lot number: H000801. Ciprofloxacin (CPFX) was from Shanghai Sanwei Pharmaceutical Co, lot number: 2001-03-012. Norfloxacin (NFLX) was from Jingzhou Jiuyang Pharmaceutical Co, lot number: 200003005. All other chemicals were purchased from Shanghai Chemical Co. SPFX, LFLX, and NFLX were suspended in 1 % sodium carboxymethylcellulose (CMC) aqueous solution. CPFX was dissolved with physiological saline. All drugs were kept to obtain an administration dose of 10 mL/kg in rats or 20 mL/kg in mice. They were dissolved in phosphate buffer solution (PBS) *in vitro*. MTT 5 g/L was dissolved in PBS.

Irradiation condition FQ have absorption peaks in the UVB (about 290 nm) and the UVA (about 340 nm) region of the solar spectrum. FQ-induced skin phototoxicity was assessed at several monochromator wavebands and found to be maximal at (365±30) nm which resemble the spectral output of the filtered PUVA source[6]. All experiments below were carried on under the condition of monochromatic irradiation at 365 nm.

Wistar rat phototoxic test Wistar rats were given SFLX, LFLX, CPFX, and NFLX at the dose of 200 mg/kg without the UVA irradiation. The vehicle was administered to each rat to detect minimal erythema dose (MED) under the UVA irradiation. Intensity of UVA was measured at 365 nm by a UVX digital radiometer (Optical and Electrical Instrument Factory, BNU, China). The rat which could not produce erythema under the irradiation of 70 % MED (Sub-MED, SMED) was regarded as the qualified rat. Such rats were divided into 12 groups, containing 8 Wistar rats (4 male and 4 female), administered by FQ at the doses of 200, 100, and 50 mg/kg, respectively. The ears of the animals were evaluated for erythema and oedema at SMED. Erythema and oedema were scored for severity on a scale of 1 to 4 by visual inspection, according to a standard scoring system commonly used to assess responses to dermal injury[7].

Balb/c mouse phototoxic test Twenty female Balb/c mice were divided into 5 groups, each group was given FQ once daily at the dose of 200 mg/kg for consecutive 7 d respectively without the UVA irradiation. One hundred twelve female Balb/c mice were divided into 14 groups (n=8). Each mouse was placed individually in partitioned plastic chambers once daily with 216 kJ/m^2 UVA irradiation for consecutive 7 d, administered by FQ at the doses of 200, 100, 50 mg/kg, and the vehicles, respectively. Assessing method was the same as the above test besides auricular thickness measured by a dial thickness gauge (peacock, Ozaki Co, Japan) at time 0 (before administration) and d 7 (immediately after the end of UVA irradiation). On d 8, the mice were sacrificed by bleeding under ether anesthesia. The auricles were fixed in 10 % buffer formalin, embedded in paraffin wax, sectioned, stained with HE, and examined histologically[8].

Chinese hamster V79 cell micronucleus test Single cell suspensions of 1.5×10^5 cells were seeded onto glass slides in 35 mm diameter culture dishes (Corning, New York, USA) and preincubated for 6 h. Prior to 4 J/m^2 UVA irradiation, the culture medium was replaced by PBS and the solvent or serial dilutions of the test chemicals were added for 50 min in the dark and further irradiated. At least 5 concentrations of FQ were tested in half-logarithm step down from their limits of solution. In order to keep the irradiation conditions identical for all slides, only 2 dishes were put side-by-side under the lamp. Non-irradiation cultures from all groups were used as controls to determine possible effects induced by FQ themselves. Following irradiation the treatment medium (PBS) was removed, the cells were washed twice and further incubated in the dark for 24 h. Cells were rinsed in 1.5 % Tri-sodium citrate 2-hydrate and fixed by methanol. The air-dried slides (24 h) were stained in 10 % of Giemsa' s solution in PBS buffer (pH 7.2) for 20 min and dried in the air. Scoring and evaluation was described as the reference[9].

NIH 3T3 MTT test Single cell suspensions of 5×10^4 cells were cultured in 96-well microtitre plates

(Nunc, Roskilde, Denmark). After 24 h, the DMEM was removed, cells were washed twice in PBS, and serial concentrations of FQ dissolved in PBS, were added. After 60 min of incubation with FQ, the cells in the microtitre plates were exposed to 50 kJ/m^2 UVA irradiation. Concurrently, second sets of plates with the same FQ were kept in the dark. After irradiation, PBS was replaced by DMEM, plates were incubated in the dark at 37 °C for 24 h. After adding MTT 5 g/L, the plates were incubated at 37 °C for 4 h, then the medium was replaced by 100 μL dimethyl sulfoxide (Me_2SO). After agitation, absorption at 570 nm was recorded on a plate reader to calculate 50 % cell growth inhibition dose (IC_{50}). The IC_{50} of test compounds was calculated from the average of triplicate cultures. The photoinhibition factor (PIF) was calculated as IC_{50} of the drug (-UV)/IC_{50} of the drug (+UV). PIF=5 for predicting phototoxic potential, PI$\geqslant$5 for phototoxic, PIF <5 for non-phototoxic[10,11].

Statistics Data were expressed as mean±SD. Statistical significance of differences between two groups was determined by *t* test. IC_{50} (95 % confidence limits) was calculated.

RESULTS

Wistar rat phototoxic test SPFX, LFLX, CPFX, and NFLX at 200 mg/kg did not show phototoxic potential without the UVA irradiation. The value of MED was in the range from 185.8 to 209.6 kJ/m^2 under the UVA irradiation. When the drugs were administered at 200 and 100 mg/kg with SMED, SPFX and LFLX showed higher phototoxic potential than that of CPFX and NFLX (Tab 1).

Balb/c mouse phototoxic test SPFX, LFLX, CPFX and NFLX at 200 mg/kg did not show phototoxic potential without the UVA irradiation. When the drugs were administered at 200, 100, and 50 mg/kg with the UVA irradiation, SPFX and LFLX showed severe phototoxicity, CPFX and NFLX were mild by visual inspection. Auricular thickness significantly increased in SPFX and LFLX groups, while CPFX and NFLX showed little change (Tab 2). Histologically, severe dermal inflammation consisting of edema, neutrophil infiltration, and hemorrhage was observed in SPFX and LFLX groups in a dose-dependent matter with the UVA irradiation, partial necrosis was also observed in SPFX group (Fig 1A). Severe hemorrhage was observed in LFLX group (Fig 1B) at 200 mg/kg. The auriculars of animals of CPFX groups at 200 mg/kg showed mild edema, neutrophil infiltration and hemorrhage (Fig 1C). NFLX groups at 200 mg/kg showed only hemorrhage (Fig 1D).

Tab 1. Mean total erythema and edema score of LFLX, SPFX, CPFX, and NFLX with SMED. ***n*****=8 rats in each group. Mean±SD.** ^{b}***P*****<0.05,** ^{c}***P*****< 0.01** ***vs*** **1 % CMC.** ^{e}***P*****<0.05,** ^{f}***P*****< 0.01** ***vs*** **physiological saline**

Dose/ mg·kg^{-1}	Mean total erythema and edema score			
	LFLX	SPFX	CPFX	NFLX
200	3.9±0.8^c	4.5±1.1^c	1.2±1.0^f	0.4±0.5
100	2.9±1.1^c	3.2±1.4^c	1.1±1.0^e	0.2±0.5
50	1.4±1.3^b	1.5±0.9^c	0.4±0.7	0.12±0.35

LFLX: lomefloxacin; SPFX: sparfloxacin; CPFX: ciprofloxacin; NFLX: norfloxacin

Tab 2. Mean total erythema and edema score and mean auricular thickness of LFLX, SPFX, CPLX, and NFLX at all doses with the UVA irradiation. ***n*****=8 mice in each group. Mean±SD.** $^\#$**Suspended in 1 % CMC.** ***Dissolved in physiological saline.** ^{b}***P*****<0.05,** ^{c}***P*****< 0.01** ***vs*** **1 % CMC.** ^{e}***P*****<0.05** ***vs*** **physiological saline.**

Drugs	Dose/ mg·kg^{-1}	Mean total erythema and edema score	Mean auricular thickness /mm
Saline	-	0.4±0.7	0.195±0.009
1% CMC	-	0.1±0.4	0.195±0.009
LFLX$^\#$	200	5.1±0.4^c	0.30±0.03^c
SPFX$^\#$		5.5±0.8^c	0.33±0.03^c
CPFX*		0.8±0.5^e	0.202±0.013
NFLX$^\#$		0.6±0.7	0.208±0.015
LFLX$^\#$	100	3.1±1.6^c	0.27±0.03^c
SPFX$^\#$		5.1±0.6^c	0.28±0.03^c
CPFX*		0.9±0.8^e	0.208±0.020
NFLX$^\#$		0.8±0.9	0.208±0.020
LFLX$^\#$	50	1.8±1.8^b	0.23±0.03^c
SPFX$^\#$		5.2±0.5^c	0.30±0.03^c
CPFX*		0.6±0.7^e	0.202±0.010
NFLX$^\#$		0.2±0.5	0.20±0.00

CMC: carboxymethylcellulose; LFLX: lomefloxacin; SPFX: sparfloxacin; CPFX: ciprofloxacin; NFLX: norfloxacin

Chinese hamster V79 cell micronucleus test V79 cells treated with UVA alone or FQ at all concentrations without the UVA irradiation did not show photogenotoxicity. The viability of cells at the highest concentration (1 mmol/L) of FQ was ≥50 % detected

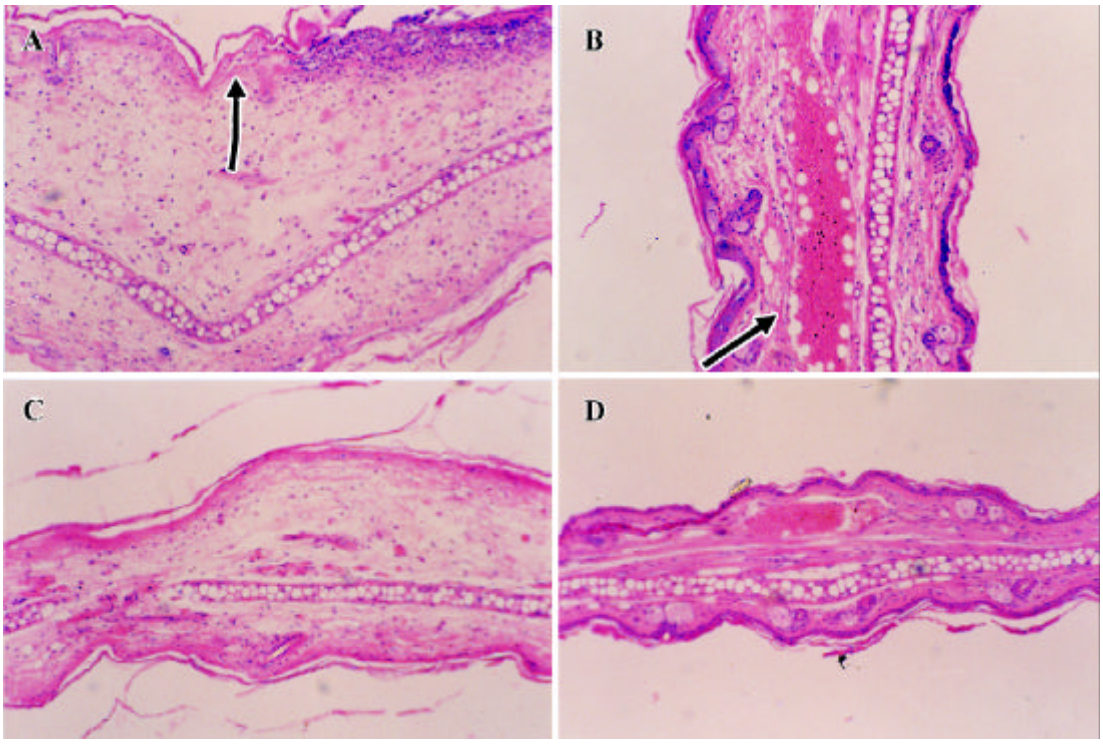

Fig 1. Auricles of Balb/c mice treated with FQ at 200 mg/kg with the UVA irradiation (HE stain, ×66). (A) Severe dermal inflammation and partial necrosis (arrow) in SPFX group; (B) Severe dermal inflammation and hemorrhage (arrow) in LFLX group; (C) Mild edema, neutrophil infiltration, and hemorrhage in CPFX group; (D) hemorrhage in NFLX group .

by crystal violet. They all increased the frequency of micronucleated cells at higher concentrations with the UVA irradiation; the frequency of micronucleated cells of SPFX and LFLX groups was higher than that of CPFX and NFLX (Tab 3).

NIH 3T3 MTT test V79 cells treated by UVA alone did not show cytotoxicity. Cytotoxicity of FQ was markedly potentiated with the UVA irradiation (Tab 4). PIF of SPFX, LFLX, CPFX, and NFLX were 8.44, 6.88, 5.35, and 5.17, respectively.

DISCUSSION

We compared the phototoxic potential of some marketed FQ *in vivo* and *in vitro*. There were good correlations between them. In all tests, LFLX and SPFX showed higher phototoxic potential, CPFX and NFLX were mild. These results demonstrate the good safety

Tab 3. Micronucleated cells induced at different concentrations of FQ in V79 cells with the UVA irradiation. *n*=6 slides. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* PBS groups.

	Micronucleated cells/%				
+UV	0.1 μmol/L	1 μmol/L	10 μmol/L	0.1 mmol/L	1 mmol/L
PBS	0.12±0.04	0.12±0.04	0.13±0.05	0.12±0.04	0.12±0.04
LFLX	0.13±0.05	0.17±0.05	1.17±0.26^{c}	5.43±0.66^{c}	5.97±0.88^{c}
SPFX	0.17±0.05	0.40±0.24^{b}	1.58±0.54^{c}	8.35±0.75^{c}	8.80±1.34^{c}
CPFX	0.12±0.04	0.17±0.05	0.62±0.23^{c}	2.27±0.41^{c}	3.65±0.58^{c}
NFLX	0.12±0.04	0.13±0.05	0.38±0.16^{c}	1.98±0.37^{c}	2.40±0.57^{c}

LFLX: lomefloxacin; SPFX: sparfloxacin; CPFX: ciprofloxacin; NFLX: norfloxacin

Tab 4. Cytotoxicities of SPFX, LFLX, CPLX, and NFLX against NIH 3T3 cells without or with the UVA irradiation.

Drug	IC_{50}/mg·L^{-1}		PIF
	-UVA	+UVA	
LFLX	169.70	24.68	6.88*
SPFX	42.97	5.09	8.44*
CPFX	361.96	67.69	5.35*
NFLX	470.10	90.95	5.17*

LFLX: lomefloxacin; SPFX: sparfloxacin; CPFX: ciprofloxacin; NFLX: norfloxacin; PIF: photoinhibition factor ; * PIF >5 shows photoxic.

profile of CPFX and NFLX in terms of phototoxicity and ensure the validation of phototoxic tests *in vitro*. Phototoxicity is dose-dependent and strengthened by the constituent at the X_8 position with a halogen of FQ.

Many FQ have been reported to cause phototoxicity in clinical practice, although the incidences were different among agents[12,13]. So, we should build a series of methods to detect their phototoxic potential. In order to ensure the validation of phototoxic test *in vitro*, we compared phototoxic potential of some marketed FQ such as SPFX, LFLX, CPFX, and NFLX *in vivo* and *in vitro*. In some reports, SPFX and LFLX showed relatively higher phototoxic potential than that of CPFX and NFLX[12,14]. In our study, the phototoxic potential of FQ *in vivo* was almost consistent with clinical observations, a number of models *in vitro* such as micronucleus test[15] and cytotoxicity for mammalian cells[11] have been used for evaluating the phototoxic potential and photogenotoxicity of FQ.

FQ have absorption peaks in the UVB (about 290 nm) and the UVA (about 340 nm) region of the solar spectrum. Different FQ has different absorption at the same waveband. In our tests, the FQ have similar λmax around 365 nm. It was reported that FQ showed a phototoxic effect, which was maximal with (365±30) nm irradiation at 24 h after phototesting. As the (365±30) nm waveband was the most spectrally similar to the output of the filtered PUVA tubes used *in vitro* and FQ-induced skin phototoxicity assessed at several monochromator wavebands was found to be maximal at (365± 30) nm, we chose (365±30) nm as irradiation source. According to USP, SPFX, LFLX, CPFX, and NFLX have the similar clinical doses when they are firstly administered to patients as a single dose. The dose ranges from 400 mg to 500 mg. We used the same dose in each test.

In Wistar rat phototoxic test, we recorded the UVA dose when a Wistar rat began to produce erythema as MED. Because each rat has different sensitivities to UVA, it was very difficult to observe the exact time. We should make sure the MED of each rat administered 1 % CMC or saline. As self-control test, it can avoid error induced by different animals. SPFX and LFLX showed obvious phototoxic potential with SMED. We can see dermal inflammation consisting erythema and edema in the group (200 mg/kg with the UVA irradiation). Compared with SPFX and LFLX, CPFX was mild and NFLX did not show different from the control. In Balb/c mouse phototoxic test, as the above test, SPFX and LFLX produced severe dermal inflammation with the UVA irradiation. From histological examination, edema, neutrophil infiltration, hemorrhage, and proliferation of epidermal cells in the auricles were observed in SPFX and LFLX at all doses with the UVA irradiation, the severe was dose-dependent. At the dose of 50 mg/kg, the phototoxic potential of LFLX group is obviously lower than that of SPFX group; it began to show phototoxicity as those of the higher doses groups 2 d after administration. CPFX showed mild phototoxicity, NFLX did not show phototoxic even at the highest dose. Maybe the dial thickness gauge used is too old; the data from the mean auricular thickness at CPFX group could not correlate with visual score and histological inspection. All data from the mean auricular thickness may be less than those we should attain. Partial necrosis was very difficult to inspect visually, we only can find scab in SPFX group at 200 mg/kg. The evaluation method is according to a standard scoring system commonly used to assess responses to dermal injury. In order to ensure the results from visual inspection and avoid mistake, histological inspections were used as an assistant method.

Reactive oxygen species participated in the mechanisms of cutaneous phototoxicity induced by FQ[16,17]. The phototoxicity of FQ was related to DNA damage caused by reactive oxygen species, especially $^1O_2$[18]. FQ may be strong photoclastogenic agents with the UVA irradiation. Micronucleus test with Chinese hamster V79 cells was used to detect FQ-induced photogenotoxicity. It also can reflect phototoxicity because FQ damaged chromosome to some extent. FQ themselves did not induce genotoxicity without the UVA irradiation. With the UVA irradiation, FQ signifi-

cantly increased the frequency of micronucleated cells at the higher concentrations. It proved that SPFX and LFLX were strong photoclastogenic agents. It is consistent with the data of DNA strand breaking activity (Comet assay)[11]. We used NIH 3T3 cells to examine the photocytotoxic effects of FQ[10]. The cytotoxicity can be enhanced by UVA irradiation. The photo-inhibition factor (PIF) of SPFX and LFLX was higher than that of CPFX and NFLX. It indicated that SPFX and LFLX had strong phototoxic potential. The potency of photocytotoxic activity of FQ was as following order: SPFX >LFLX >CPFX >NFLX, according to the PIF.

The extent of skin phototoxicity strongly differs from one FQ to another one. Phototoxicity of FQ *in vivo* and *in vitro* may be connected with structure-activity relationships. The constituent at X_8 position of FQ is mainly responsible for phototoxic potential, FQ with a halogen substitution have the highest potential such as SPFX, LFLX, and Bayer Y3118, while substitution with a methoxy group effectively reduces phototoxicity[19-21]. Substitution with a methoxy group or other group at X_8 has a low phototoxic potential when administered to healthy subjects[22].

We compared some market FQ under different UVA irradiation *in vivo* and *in vitro*, SPFX and LFLX showed obvious phototoxic potential *in vivo* and *in vitro*. CPFX and NFLX showed obvious phototoxic potential at higher doses with the UVA irradiation *in vitro*. It is suggested that CPFX and NFLX have a low risk for phototoxicity than that of SPFX and LFLX, which are reported to have strong phototoxicity. In our study, the phototoxic potential of FQ was as the following order: SPFX>LFLX>CPFX>NFLX. The phototoxic potential results *in vivo* are correlated with those of clinical observation. Balb/c mouse phototoxic test was more sensitive than that of Wistar rats. Because the good correlation between the results of phototoxic tests *in vivo* and *in vitro*, we can ensure the validation of phototoxic tests *in vitro* and apply them to detect the phototoxic potential of some new FQ in some feasible conditions.

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