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IN VIVO REVERSAL OF MULTIDRUG RESISTANCE BY TWO NEW DIHYDROPYRIDINE DERIVATIVES, S16317 AND S16324

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Two new dihydropyridine derivatives with low calcium channel affinity, S16317 and S16324, were found to fully overcome multidrug resistance in vitro. These two compounds increased doxorubicin cytotoxicity on the human COLO 320DM cell line and completely reversed the vincristine resistance of murine P388/VCR cells. In vivo, S16324 administered p.o. (200 mg/kg on days 1 to 4) or i.p. (50 mg/kg on days 1, 5, 9) in combination with vincristine (i.p.) restored the antitumor activity of vincristine in P388/VCR-bearing mice. S16317 showed a reversing activity when administered p.o., i.v. (days 1 to 4) or i.p. (days 1, 5, 9) at the same dose (25 mg/kg), suggesting a remarkable bioavailability. Moreover, these two compounds potentiated the antitumor activity of vincristine in the sensitive P388 leukemia, increasing the number of long-term survivors. These results suggest that combination chemotherapy using S16317 or S16324 would be effective not only in circumventing multidrug resistance but also in preventing the emergency of a population of resistant tumor cells in sensitive tumors.

It is widely accepted that the majority of agents that reverse multidrug resistance (MDR) act through their ability to inhibit the activity of P-glycoprotein (P-gp), an energy-dependent efflux pump (1, 2), inducing an increase of intracellular concentration of cytotoxic drugs (3, 4). The reversing activity of these compounds is thus dependent, in part, on their interaction with the P-gp (5, 6). Among the various compounds described as sensitizing agents, numerous calcium channel blocker derivatives have been reported to reverse MDR (7). However, while many of these pharmacological agents have been found to completely overcome drug resistance in vitro, the number of reports showing such a phenomenon in vivo is more limited (8–11). The lack of activity of chemosensitizers in vivo results principally from the difficulty of maintaining active doses without causing serious side effects (7, 9). Indeed, the clinical use of these compounds is limited by their strong vasodilatator effect due to their primary pharmacological activity (12). Consequently, potent MDR-reversing agents with low calcium channel blocking activity would be of value in cancer chemotherapy.

In order to find novel drugs active for the treatment of cardiovascular diseases, new racemic 1,4-dihydropyridine derivatives were evaluated in our institute for their L-type Ca^{2+} channel affinity (13). Among the compounds of this series characterized by a long side chain on position 2 of the dihydropyridine ring, the enantiomers having the (+) configuration were found to have less calcium channel affinity and to be less potent in inhibiting L-type Ca^{2+} current than the corresponding (−) isomers (14). In vitro, certain racemic derivatives were found to slightly but significantly sensitize MDR cell lines to cytotoxic drugs, the both enantiomers having similar activity. To optimize these results it was necessary to enhance MDR reversing

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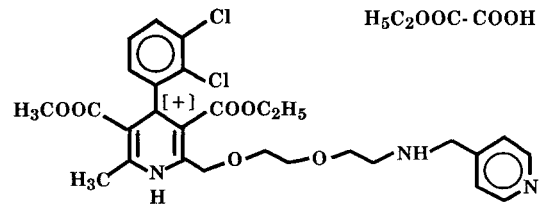
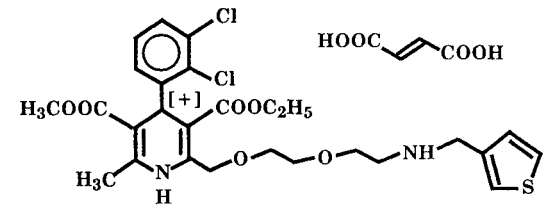
activity while maintaining a low calcium channel affinity. We thus synthesized about 50 new 1,4-dihydropyridine derviatives with (+) configuration. Screening in vitro using the highly resistant rodent cell line DC-3F/AD resulted in the selection of S16317 and S16324.

Recently, a number of dihydropyridine derivatives with low calcium channel-blocking activity have been described to efficiently overcome MDR in vitro, and to sensitize MDR tumors in the mouse model (5, 11). In most of these in vivo studies, the reversing activity was evaluated after administration of the modulators and anti-tumor drugs to the animals by the same route, i.e. intraperitoneally (i.p.) (5, 15). This model is, however, quite artificial and is probably of little predictive value for the clinic. Consequently, taking into account the good known bioavailability of our compounds, we decided to evaluate them in vivo using different routes of administration. We now report that S16317 and S16324 were highly efficient both in potentiation of doxorubicin (ADR) and vincristine (VCR) cytotoxicity on human and murine cell lines in vitro, and in reversal of VCR resistance in the murine P388/VCR leukemia model in vivo.



S 16317



S 16324

Fig. 1. Chemical structures of 1,4-dihydropyridine derivatives S16317 and S16324.

Material and Methods

Drugs. Verapamil (VRP) was obtained from the Sigma Chemical Co. (St. Louis, MO); doxorubicin (ADR) was from Roger Bellon (Neuilly, France), vincristine sulfate (VCR) from Lilly (France) and cyclosporin A (CsA) from Sandoz (Bale, Switzerland). S16317 and S16324 were synthesized at the Servier Research Institute (Fig. 1).

Dihydropyridine binding studies to calcium channels. The experiments were performed on pig aortic smooth muscles microsomes as previously described (16). Calcium channel affinities of nifedipine, S16317 and S16324 were evaluated by studying the inhibition of [^{3}H] PN 200–110 binding.

Cell lines. The murine leukemia P388/VCR-20 was established from P388 and was 94-fold resistant to VCR. COLO 320DM (an intrinsically resistant human colon adenocarcinoma) was from the American Type Culture Collection (Rockville Pike, MD). All cell lines were grown in RPMI medium 1640 supplemented by 10% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM Hepes buffer, pH 7.4. The P388/VCR-20 cell line was cultured in the presence of 20 nM VCR until one week prior to the beginning of the experiments. The cross resistance and the P-gp overexpression of P388/VCR-20 and COLO 320DM have been previously described (17, 18).

Cytotoxicity. Cytotoxicity was measured by the microculture tetrazolium assay as described (18, 19). The drug concentration inhibiting 50% of the proliferation with respect to untreated cells (IC_{50}), was measured after continuous exposure (4 doubling times) to both the cytotoxic drug and the reversal agent. The fold resistance was defined as the ratio: IC_{50} in resistant cell line / IC_{50} in sensitive line, for a given cytotoxic drug. The reversing activity was expressed as fold reversion (F. REV), F. REV = IC_{50} cytotoxic drug alone / IC_{50} cytotoxic drug + modulator. The average cytotoxicity of S16324 was similar to that of CsA (IC_{50} = 3.0 and 3.2 μM respectively); S16317 and VRP were less cytotoxic (IC_{50} = 8.0 and 39.0 μM respectively).

Doxorubicin uptake studies. The dose-dependent effect of the modulators was studied essentially as described (17). COLO 320DM cells, ($5x10^5$/ml), were incubated with 50 μM ADR at 37°C for 5 h with 0.5 to 5 μM reversal agents. The mean ADR fluorescence was measured at 4°C by flow cytometry as previously described (17) and results are expressed as the increase in the mean ADR fluorescence of treated cells compared with the mean ADR fluorescence of untreated cells. Results are given for three independent experiments.

Mice and tumors. Inbred (DBA2) and hybrid female F1(C57B16 × DBA/2) mice weighing 20–22 g were purchased from Iffa credo (France). They were maintained under specific pathogen-free conditions. The P388, and VCR-resistant P388/VCR, murine leukemia cell lines were provided by the National Cancer Institute (Bethesda, Maryland, USA) and were maintained by weekly i.p. passages in DBA2 mice. Resistance of P388/VCR was retained naturally without any further administration of VCR.

Antitumor activity. S16317 was dissolved in 5% ethanol/H_2O. S16324 was dissolved in HCl (one molar equivalent), then diluted in 5% ethanol/H_2O. VCR was dissolved in

sterile 0.15 M NaCl. F1 mice were inoculated intraperitonealy (i.p.) with 10^6 P388 or P388/VCR cells on day 0. Both drugs were administered intravenously (i.v.), per os (p.o.) or i.p. in a volume of 0.1 ml/10g body weight, either alone or in a combination with VCR (i.p.), the modulator being given 30 to 60 min before VCR. Mice survival was observed during the experimental period of 60 days. Antitumor activity was expressed as: T/C (%) = (MTS of treated group/MTS of control group) × 100; T/V (%) = (MTS of modulator + VCR treated group/MTS of VCR treated group) × 100; MTS = Median time of survival.

Results

Binding to calcium channels. The binding of S16317 and S16324 to calcium channels was compared with that of nifedipine, a dihydropyridine generally used as reference, in pig aortic smooth muscles microsomes. The Ki values obtained for S16317, S16324 and nifedipine were 55 10^{-8} M, 100 10^{-8} M and 2.5 10^{-8} M respectively. The affinities of S16317 and S16324 for calcium channels were thus 22- and 40-fold lower than that of nifedipine.

In vitro reversal of ADR and VCR resistance. Fig. 2 shows the fold reversal as a function of non-cytotoxic concentrations of S16317, S16324, VRP and CsA (percentage of cellular viability > 75%) on the COLO 320DM cell line. A dose-dependent reversion of resistance was observed with the 4 reversal agents. S16317 was the most efficient compound with, at 1 μM, a reversion 2.3 times higher than that induced by VRP. At this concentration, S16324 and CsA were equiactive and displayed a reversing activity similar to that measured with 5 μM VRP. Table 1 shows the effect of 2.5 μM reversal agents on VCR cytotoxicity for the sensitive P388 and the resistant P388/VCR-20 cell lines. While 2.5 μM VRP had no significant effect on VCR cytotoxicity in P388 cells, the same concentration of S16317, S16324 and CsA enhanced 6, 7.7 and 3.9 times respectively the ability of VCR to inhibit cell proliferation. On P388/VCR-20 cells, 2.5 μM CsA, S16317, and S16324 fully overcame the VCR resistance with F. REV. = 302.6, 221.9, and 256.1 respectively. On this cell line, 2.5 μM VRP induced only a partial reversion with F. REV. = 9.3.

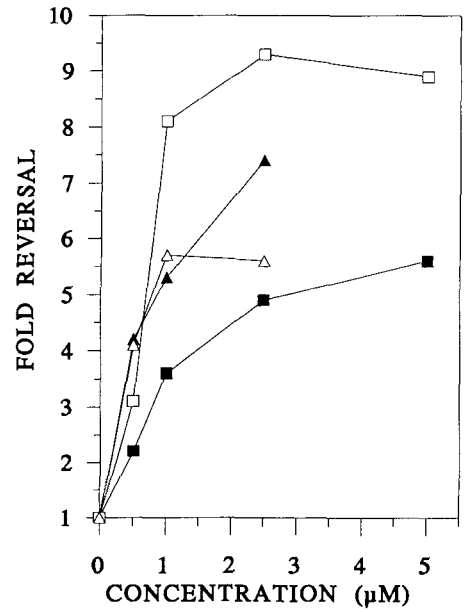


Fig. 2. Fold reversal of multidrug resistance by various concentration of S16324 (△), CsA (▲), S16317 (□), and VRP (■). COLO 320DM cells were exposed for 4 doubling times to ADR, with or without the modulators. The fold reversal is the ratio: IC_{50} (cytotoxic drug alone)/IC_{50} (cytotoxic drug + modulator).

Effect of S16317 and S16324 on ADR accumulation. As for the studies of reversion, experiments of ADR accumulation were performed on the COLO 320DM cell line. Fig. 3 shows the increase of ADR accumulation as a function of reversal agent concentration. The four tested compounds induced a dose dependent increase in ADR accumulation. CsA was the most active compound with, at 1 μM, an activity 2.2 times higher than that of VRP. S16317 and S16324 also show a good activity at 1 and 2.5 μM respectively, the increase in ADR accumulation being similar to that measured with 5 μM VRP.

In vivo reversal of drug resistance by S16317 and S16324. The ability of S16317 and S16324 to potentiate

Table 1

Effect of 2.5 μM modulators on VCR cytotoxicity

Cell lines	Without modulators	With 2.5 μM modulator			
		CsA	VRP	S16317	S16324
P388	0.54	0.14	0.22	0.09	0.07
	(F. rev)	(3.9)	(2.5)	(6.0)	(7.7)
P388/VCR-20	33.29	0.11	3.57	0.15	0.13
	(F. rev)	(302.6)	(9.3)	(221.9)	(256.1)

Cells were exposed for 4 doubling times to VCR, with or without the modulators. Fold Reversal (F. rev) = IC50 (VCR alone)/IC50 (VCR + modulator).

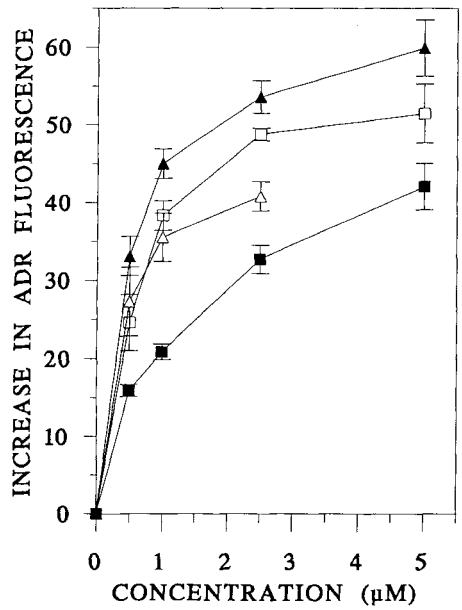


Fig. 3. Effect of S16317 (□), CsA (▲), S16324 (△), and VRP (■) on ADR accumulation. COLO 320DM cells were incubated with ADR in the presence of modulators for 5 h, and ADR fluorescence was measured by flow cytometry. Results are expressed as the increase in the mean fluorescence of treated cells compared with the mean fluorescence of untreated cells. Bars represent S.E. of 3 independent experiments.

the antitumor activity of VCR in the resistant P388/VCR leukemia model is presented in Tables 2 and 3. VCR was administered i.p., and the two dihydropyridines i.v., i.p. or p.o. Whatever the route of administration, the treatment of P388 or P388/VCR-bearing animals with S16317 and S16324 alone at the maximum doses did not alter their survival time as compared to the control (T/C values 94–112%) (Tables 2, 3 and 4). When S16317 and S16324 were administered i.p., the optimal schedule was found to be co-administration of modulator and cytotoxic drug at days 1, 5 and 9. In this schedule, VCR alone at the dose of 0.5 mg/kg showed high activity against the sensitive P388 tumor (T/C = 211%, Table 2) but only slight therapeutic effect against the resistant P388/VCR (T/C = 134%), as expected. Higher doses of VCR (up to 1.5 mg/kg) did not improve this low antitumoral effect on P388/VCR (data not shown). S16317 administered i.p. at 25 mg/kg in association with VCR was shown to completely reverse the resistance of P388/VCR, the increase in life-span (T/C = 262%, Table 2) being even superior to that observed for P388-bearing mice treated with VCR alone (T/C = 211%, Table 2). A markedly improved therapeutic effect was also noted when VCR was co-administered i.p. with S16324 at the dose of 50 mg/kg (T/C = 193%). For the other routes of modulator administration (p.o., i.v.), the optimum schedule was a treatment over 4 days (day 1 to 4), VCR (0.25 mg/kg) being administered i.p. (Table 3). As indicated in Table 3, VCR administered i.p. daily for 4 days (0.25 mg/kg) showed a low activity in P388/VCR bearing mice (T/C value 133%), this activity remaining the same with doses of VCR up to 1 mg/kg (data not shown). S16324 administered p.o. and S16317 administered p.o. or i.v. were again effective in enhancing, in a dose dependent way, the therapeutic effect of VCR in P388/VCR bearing mice. The T/C value obtained at 200 mg/kg of S16324 (T/C = 209%) was comparable with that observed in P388-bearing mice treated with VCR alone (T/C = 204%, Table 3), indicating complete circumvention of resistance by combination VCR-S16324 therapy. S16317 administered i.v. (25 mg/kg) in association with VCR showed similar reversal activity to that observed when the modulator was administered p.o. (T/C values 186% and 180%, respectively) (Table 3).

In vivo activity of S16317 and S16324 in the sensitive P388 leukemia model. We further examined if the two

Table 2

In vivo activity of S16317 and S16324 in P388/VCR tumor-bearing mice

Drugs and route of administration			Dose (mg/kg/day)	Median survival time	Median T/C (%)	T/V (%)
VCR		IP	0.5	11.7	134	100
Control		–	–	8.7	100	–
	S16324	IP	75	8.5	98	–
VCR +	S16324	IP	75	12.0	138	103
VCR +	S16324	IP	50	16.8	193	144
VCR +	S16324	IP	25	14.3	164	122
	S16317	IP	25	9.5	109	–
VCR +	S16317	IP	25	22.8	262	195
VCR +	S16317	IP	12.5	15.0	172	128

Effect of i.p. administration of S16317 and S16324 on antitumor activity of VCR on P388/VCR-bearing mice. Each group consisted of 8–10 mice. S16317, S16324 and VCR were given at days 1, 5, 9. In the absence of modulator, T/C value for VCR (0.5 mg/kg day 1, 5, 9) on sensitive P388-bearing mice was 211% (range 197–219).

Table 3

In vivo activity of S16317 and S16324 in P388/VCR tumor-bearing mice

Drugs and route of administration			Dose (mg/kg/day)	Median survival time	Median T/C (%)	T/V (%)
VCR		IP	0.25	11.3	133	100
Control		–	–	8.5	100	–
	S16324	PO	200	8.8	104	–
VCR +	S16324	PO	200	17.8	209	157
VCR +	S16324	PO	150	17.3	204	153
VCR +	S16324	PO	100	15.3	180	135
	S16317	PO	25	9.5	112	–
VCR +	S16317	PO	25	15.3	180	135
VCR +	S16317	PO	12.5	15.0	176	132
	S16317	IV	25	8.0	94	–
VCR +	S16317	IV	25	15.8	186	140
VCR +	S16317	IV	12.5	15.1	178	134

Effect of p.o. or i.v. administration of S16317 or S16324 on antitumor activity of VCR on P388/VCR-bearing mice. Each group consisted of 8–10 mice. S16317, S16324 and VCR were given daily from day 1 to day 4. T/C value for VCR (0.25 mg/kg, day 1–4) on sensitive P388-bearing mice was 204% (range 184–226).

Table 4

In vivo activity of S16317 and S16324 in P388 tumor-bearing mice

Drugs and route of administration			Dose (mg/kg/day)	Median survival time	Median T/C (%)	Survivors at day 60
VCR		IP	0.25	23.3	226	0/16
Control		–	–	10.3	–	–
	S16324	PO	200	10.5	102	0/8
VCR +	S16324	PO	200	> 60.3	> 585	5/8
	S16317	PO	25	10.5	102	0/8
VCR +	S16317	PO	25	42	408	1/8
VCR +	S16317	PO	12.5	56	544	3/8

Effect of p.o. administration of S16317 and S16324 on antitumor activity of i.p. VCR on P388/VCR-bearing mice. S16317, S16324 and VCR were given daily from day 1 to day 4.

dihydropyridine derivatives were also effective in enhancing the therapeutic effect of VCR in P388-bearing mice (Table 4). Daily p.o. administration of S16317 (25 mg/kg) and S16324 (200 mg/kg) over 4 days had no antitumoral effect in P388/sensitive-bearing mice (T/C = 102%). In this schedule, VCR administered i.p. at 0.25 mg/kg showed good antitumoral activity (T/C = 226%). When S16324 (200 mg/kg) and S16317 (25 mg/kg) were associated with VCR (0.25 mg/kg), increases in life span, over that obtained with treatment of P388-bearing animals with VCR alone, were obtained (T/C values 408% and > 585% respectively). Moreover, the combined treatment of each modulator with VCR resulted in an increased number of mice surviving up to 60 days (Table 4). Fig. 4 shows the results of one such experiment. The difference observed between the survival curves of the control group and the VCR-treated group was found to be highly significant when analyzed by the log-rank test ($p < 0.001$). A significant difference was also noted when the survival curves of

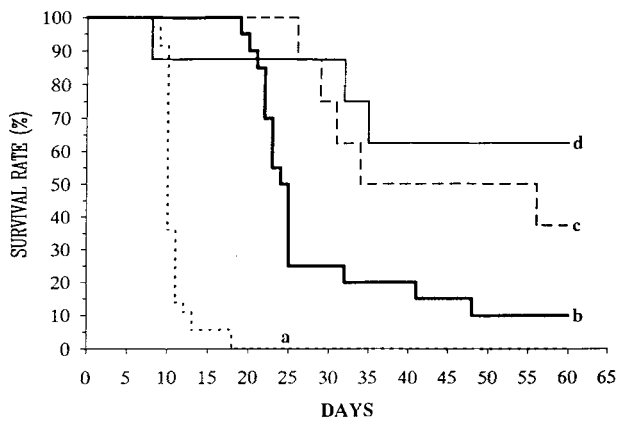


Fig. 4. Effect of S16317 and S16324 on antitumor activity of VCR in P388-bearing mice. S16317 and S16324 were given p.o. and VCR was given i.p. daily from day 1 to 4. Curves a: control; b: VCR (0.25 mg/kg); c: VCR (0.25 mg/kg) plus S16317 (12.5 mg/kg); d: VCR (0.25 mg/kg) plus S16324 (200 mg/kg). Each group consisted of 8 to 10 mice.

VCR and (S16324 + VCR) or (S16317 + VCR) treated groups were compared ($p < 0.004$). As a result, S16317 and S16324 clearly potentiated the antitumor activity of VCR in the sensitive P388-tumor model.

Discussion

The focus of our research was to find 1,4-dihydropyridine derivatives with low calcium channel blocking activity and high potency in reversal of MDR. The screening of about 50 compounds synthesized in our laboratory resulted in the selection of S16317 and S16324. Their low channel blocking activity may explain the relatively low toxicity of S16317 and S16324 observed in the in vivo experiments. For example, whereas S16317 was highly active at the doses of 12.5 and 25 mg/kg, the administration of this compound alone at 200 mg/kg p.o. daily during 4 days caused no significant weight loss (data not shown). However, further studies are needed to carefully monitor the effects of these compounds, at active dosages, on the cardiovascular system, in view of an eventual clinical development.

In vitro, S16317 and S16324 completely reversed the resistance of P388/VCR cells to VCR and increased ADR cytotoxicity on the COLO 320DM cell line. For this human cell line, their reversing activity was dose-dependant and superior to that of VRP, S16317 being the most potent and active compound. Furthermore, S16317 and S16324 induced an increase in ADR accumulation in COLO 320DM cells, suggesting an inhibition of the P-gp-mediated efflux of ADR. Nevertheless, other unknown mechanisms cannot be excluded since S16317 and S16324 were also able to significantly enhance VCR cytotoxicity on the sensitive P388 cell line.

In vivo, most previously published experiments have been performed using the same route for both tumor inoculation and administration of chemotherapeutic agents, for example, i.p. (5, 15). However, various routes of administration of reversal agents should be considered in the conception of valid experimental therapeutic models. In the present study we demonstrated that S16317 and S16324 administered i.p. or p.o. (or i.v. for S16317) in combination with VCR administered i.p. restored the antitumor activity of VCR in mice inoculated i.p. with P388/VCR leukemia. S16324 showed higher activity when administered p.o. compared to i.p.. S16317 (25 mg/kg) restored the antitumoral activity of VCR whatever the route of administration (i.p., p.o. or i.v.), suggesting a remarkable bioavailability of our compound compared to other dihydropyridine derivatives (5, 10, 20).

Significant therapeutic effects are also observed in the sensitive P388 model. The combination of S16317 or S16324 and VCR resulted in a more than 200% increase of life prolongation and also an increase of the number of long-term survivors compared to that obtained with VCR treatment alone (Fig. 3). Other 1,4-dihydropyridine derivatives, have also previously been shown to potentiate the activity of anticancer drugs on sensitive experimental tumors (10, 11, 20). Such an important therapeutic effect against the sensitive cells is in accordance with results obtained in vitro, where S16317 and S16324 were found to enhance the cytotoxic effect of VCR (Table 1). The modulators might also be effective in vivo in preventing the emergency of a population of resistant tumor cells in the sensitive tumor (3), or in modifying the pharmacokinetics of cytotoxic drugs as described in the case of CsA (21). Further studies will be required for the continued exploration of the therapeutic potential of S16317 and S16324, particularly on human tumor xenograft models.

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