Effects of 3,3′-Dihydroxy-α,β-diethylstilbene and 3,3′,4,5′-Tetrahydroxystilbene on Microtubule Assembly *in Vitro*, Aneuploidy Induction, and Cellular Microtubule and Actin Networks

Yumiko SAKAKIBARA,[a] Izumi SAITO,[a] Taiko ODA,[a] Hiroshi TSUJIBO,[b] Yoshihiko INAMORI,[b] and Yoshihiro SATO*,[a]

Kyoritsu College of Pharmacy,[a] Shibakoen 1-chome, Minato-ku, Tokyo 105, Japan and Osaka University of Pharmaceutical Sciences,[b] Kawai, Matsubara-shi, Osaka 580, Japan. *Received December 11, 1991*

We examined the inhibitory activities of 3,3′-dihydroxy-α,β-diethylstilbene (DDS) and 3,3′,4,5′-tetrahydroxystilbene (THS) on microtubule assembly *in vitro* and their effects on chromosome number and cellular microtubule networks in Chinese hamster V79 cells. DDS showed half the inhibitory activity of diethylstilbestrol (DES) on microtubule assembly *in vitro*, while THS had none of the inhibitory activity. DDS induced tetraploid at 30 μM, whereas THS was found to be inactive. Furthermore, DDS disturbed cellular microtubule networks at 100 μM.

We also examined the effects of DES, DDS and THS on cellular actin networks in mouse BALB 3T3 cells. DES induced a change of actin stress fiber distribution and THS had similar activity, while DDS showed no activity.

Keywords microtubule assembly; aneuploidy; 3,3′-dihydroxy-α,β-diethylstilbene; 3,3′,4,5′-tetrahydroxystilbene; diethylstilbestrol; Chinese hamster V79 cell; mouse BALB 3T3 cell; immunofluorescence; actin

Introduction

We reported the inhibitory activities of diethylstilbestrol (DES; Fig. 1),[1] a synthetic estrogen as well as a carcinogen, and DES analogues[2] on microtubule assembly *in vitro*. Recently, we also reported the effects of DES and its methylethers on chromosome number and the cellular microtubule architecture of Chinese hamster V79 cells.[3]

Inamori, one of the present authors, reported that 3,3′,4,5′-tetrahydroxystilbene (THS; Fig. 1) isolated from the heartwood of *Cassia garetiana* CRAIB. (Leguminosae) showed strong antifungal, antimicrobial, phytogrowth-inhibitory and ichthyotoxic activities, and coronary vasodilator action on isolated guinea pig hearts.[4] Those activities of oxystilbene derivatives were subsequently examined in an attempt to find more active substances, and it was reported that DES and 3,3′-dihydroxy-α,β-diethylstilbene (DDS; Fig. 1), an isomer of DES, have stronger activities than THS.[5]

In this work, we examined the inhibitory activities of DDS and THS on microtubule assembly *in vitro*; the effects of these compounds on chromosome number and the cellular microtubule architecture of Chinese hamster V79 cells were compared with those of DES. We also examined the effects of DES, DDS and THS against actin stress fibers in mouse BALB 3T3 cells.

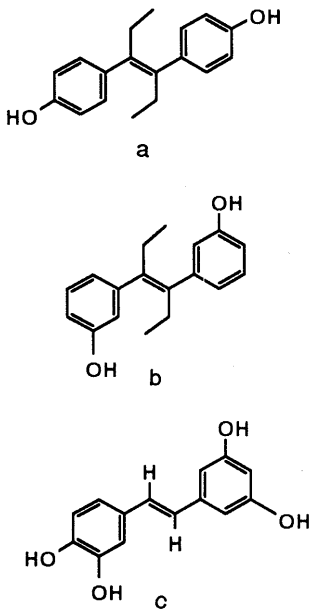

Fig. 1. Structures of a, Diethylstilbestrol; b, 3,3′-Dihydroxy-α,β-diethylstilbene; and c, 3,3′,4,5′-Tetrahydroxystilbene

Materials and Methods

Reagents DDS,[6] THS[7] and DES [Tokyo Chemical Industry Co., Ltd. (Tokyo)] were used for the activity test. Adenosine triphosphate (ATP) and guanosine triphosphate (GTP) were obtained from Yamasa Shoyu Co., Ltd. (Choshi), and the materials for electron microscopy were from Nissin EM Co., Ltd. (Tokyo). For cell culture experiments, fetal bovine serum (FBS) was obtained after lot-checking from GIBCO Laboratories (Grand Island, NY), dimethyl sulfoxide (DMSO) was purchased from the Pierce Chemical Co. (Rockfield, IL) and colcemid from GIBCO. All other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka).

Preparation of Microtubule Proteins The preparations of microtubule proteins were performed as described[2b] from porcine brains by two cycles of temperature-dependent assembly-disassembly.[8] Concentration of microtubule proteins was determined by the method of Lowry *et al.*[9] using bovine serum albumin as the standard.

Assembly Assay The effects of the test compounds on microtubule proteins at 37 °C were determined by turbidity measurement[10] at 400 nm using a UVIDEC 430B double-beam spectrometer equipped with a thermostatically controlled cell holder. Microtubule proteins were adjusted to a concentration of 3 mg protein/ml.

Electron Microscopy The polymers of microtubule proteins were observed by electron microscope as described.[2b] Samples were fixed by addition of 9 volumes of the buffer for assembly containing 1% glutaraldehyde, then negatively stained with 1% uranyl acetate solution and air-dried. Specimens were examined on a JEOL 200 CX electron microscope at 100 kV.

Cell Culture Chinese hamster V79 cells and mouse BALB 3T3 cells were grown in monolayer culture in Eagle's minimum essential medium (MEM) [Nissui Pharmaceutical Co., Ltd. (Tokyo)] with 10% heat-inactivated FBS. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

Relative Plating Efficiency The relative plating efficiencies in the presence of different concentrations of drugs were determined as described.[3] Cells were seeded in 200/60-mm petri dishes [Falcon; Becton Dickinson & Company (Lincoln Park, NJ)] in 4 ml of MEM supplemented with 10% FBS. At 24 h after seeding, drugs dissolved in DMSO were added to the culture for 48 h. Then, the medium was replenished, and the culture was continued for 48 h without drugs. The dishes were fixed with methanol and stained with 7% Giemsa solution. The number of colonies (> 50 cells/colony) was counted under a dissecting microscope. DMSO alone was added to control culture.

Determination of Chromosome Number and Chromosome Aberrations Five-tenths—2×10^5 cells were seeded in 10-mm petri dishes in 10 ml of MEM with 10% FBS and incubated overnight. After drug treatment for

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24 h, the medium was changed and the culture was treated with colcemid (0.2 μg/ml) for 2 h. After trypsinization, the cells were treated with 0.075 M KCl at room temperature for 25 min, fixed in Carnoy's solution (methanol : acetic acid, 3 : 1) and spread on glass slides to air-dry. The specimens were stained with 2% Giemsa solution in 1/15 M Sörensen phosphate buffer (pH 6.8) for 5 min. Over 100 metaphases were counted to examine the number of chromosomes and chromosome aberrations.

Indirect Immunofluorescence for Microtubules Cells (800 cells/40 μl were placed on one of several 8-well multitest slides [Flow Laboratories, Ltd. (Irvine, Scotland)], incubated for 24 h at 37 °C in a wet box, and then treated with a drug. Twenty μl of medium per well was removed and 20 μl medium which contained twice the concentration of drug dissolved in DMSO was added.

The cell fixing and staining methods were performed as described[3] according to the method of Wheeler *et al.* with some modifications.[11] Cells on slides were fixed with 3% formaldehyde in phosphate-buffered saline (PBS), permeabilized with cold methanol, and air-dried. Cells were pretreated with 1% Triton X-100 in PBS and 2% dry milk in PBS, and then incubated for 1 h with mouse monoclonal anti-β-tubulin antibody [Amersham Laboratories (Buckinghamshire, England)] at 37 °C. Following three 5 min rinses in PBS containing 2% dry milk, cells were further incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG_1 [Binding Site, Ltd. (Birmingham, England)] at 37 °C. Following rinses in PBS containing 2% dry milk and then in PBS, the wet slides were mounted in FA mounting fluid at pH 9 [Difco Laboratories (Detroit, MI)]. The slides were examined and photographed with Fuji color super HG 400 (ASA 400) film under a 100 × Olympus DApo VU lens, using an Olympus BHS-RFK fluorescence microscope with an Olympus PM-10ADS autophotography system.

Indirect Immunofluorescence for Actin Stress Fibers Because Chinese hamster V79 cells did not give as clear cytoplasmic actin networks when observed by indirect immunofluorescence, mouse BALB 3T3 cells were used for this experiment. The cell seeding and drug treatment were performed by the method used for the observation of microtubule networks.

Cell fixing and staining were performed by the methods of Danowski[12] with some modifications. Cells on slides were fixed at room temperature for 10 min with 2% formaldehyde and 0.1% glutaraldehyde in PBS, then treated for 2 min with 1% Triton X-100 in PBS. Cells were preincubated for 30 min with 2% dry milk in PBS, and then further incubated for 30 min at 37 °C with 20 μl per well of rhodamin-conjugated phalloidin solution [Molecular Probes Inc. (Eugene, OR)]. Slide observation was similar to that for microtubule networks.

Results and Discussion

Effects of DDS and THS on Microtubule Proteins We examined the effects of DDS and THS on microtubule polymerization *in vitro*, and their turbidimetrical curves are shown in Fig. 2. In comparison with the previous data,[2a] the inhibitory activity of DDS was half that of DES and similar to that of *meso*-hexestrol. In the presence of 50 μM DDS, many short microtubules were observed by electron microscopy. At 100 μM DDS, only ribbon structures were observed; these ribbon structures were similar to those induced by *meso*- and *dl*-hexestrol as described in the previous paper[2a] (data not shown).

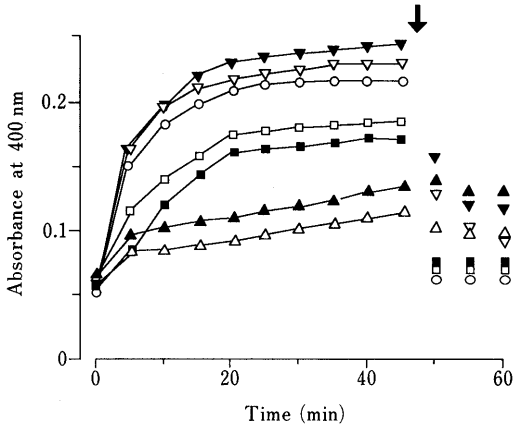

Fig. 2. Turbidimetric Analysis of Assembly-Inhibition of Microtubules by DDS and THS at 37 °C

Test compounds were added to microtubule proteins (3 mg/ml) at 0 min. The final concentrations of drugs were: ○, control; □, 25 μM DDS; ■, 50 μM DDS; ▲, 100 μM DDS; △, 200 μM DDS; ▽, 100 μM THS; ▼, 200 μM THS. Arrow indicates the temperature shift down to 0 °C.

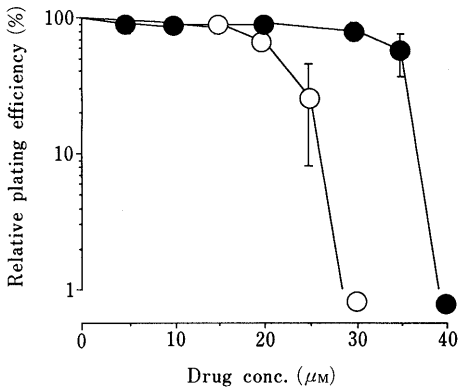

Fig. 3. Relative Plating Efficiencies of Chinese Hamster V79 Cells Treated with DDS and THS for 48 h with Various Concentrations of Drugs

○, DDS; ●, THS.

TABLE I. Incidence of Aneuploidy and Chromosome Aberrations in V79 Cells Following 48-h Treatment with DDS and THS

Drug	Conc. (μM)	% diploid cells	% heteroploid cells			Aberrant metaphases (%)	Type of aberration (%)								
			Near di-	Near tetra-	Near octa-		G	ICG	E	O	B	D	P	Ero	R
Control		94	6	0	0	0	0	0	0	0	0	0	0	0	0
DDS	10	93	7	0	0	0	0	0	0	0	0	0	0	0	0
	15	87	11	2	0	0	0	0	0	0	0	0	0	0	0
	20	87	11	2	0	0	0	0	0	0	0	0	0	0	0
	25	79	20	1	0	0	0	0	0	0	0	0	0	0	0
	30	16	18	62	4	28	3	1	0	0	0	15	3	7	2
THS	5	90	8	2	0	0	0	0	0	0	0	0	0	0	0
	10	96	1	3	0	0	0	0	0	0	0	0	0	0	0
	20	95	3	2	0	0	0	0	0	0	0	0	0	0	0
	30	91	4	5	0	0	0	0	0	0	0	0	0	0	0
	35	87	8	5	0	0	0	0	0	0	0	0	0	0	0
	40	82	15	3	0	0	0	0	0	0	0	0	0	0	0

Chromosome number of diploid cells: 22 or 21, of near diploid cells: 18—28, of near tetraploid cells: 29—50, of near octaploid cells: more than 51. G: gap, ICG: isochromatid gap, E: exchange, O: 0-ring, B: break, D: dicentric, P: pulverization, Ero: erosion, R: ruffling.

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Turbidity measurement showed, however, that THS did not affect microtubule assembly. By electron microscopy, some irregular aggregates were observed other than microtubules; but at 200 μM THS, long microtubules were observed similar to control without drug. We therefore concluded that THS had no inhibitory activity on microtubule assembly *in vitro*.

Cytotoxicities of DDS and THS in V79 Cells Figure 3 shows the relative plating efficiency of V79 cells at various concentrations of DDS and THS. Treatment with 20 μM DDS or 35 μM THS for 48 h suppressed the colony formation in 25% the cells, while that with 30 μM DDS or 40 μM THS suppressed more than 99% of the cells. The IC_{50} (the concentration required for 50% inhibition of colony formation; DES = 13 μM[3]) was 22 μM for DDS and 37 μM for THS.

Dose Effects of DDS and THS on Aneuploidy and Chromosome Aberration V79 cells were incubated with various concentrations of DDS and THS for 48 h and then treated with colcemid for 2 h. As summarized in Table I, 30 μM DDS induced aneuploidy through chromosome loss and gain although untreated V79 cells had 22 or 21 chromosomes; the frequency of aneuploidy by 30 μM DDS was comparable with that by 15 μM DES, while 40 μM THS

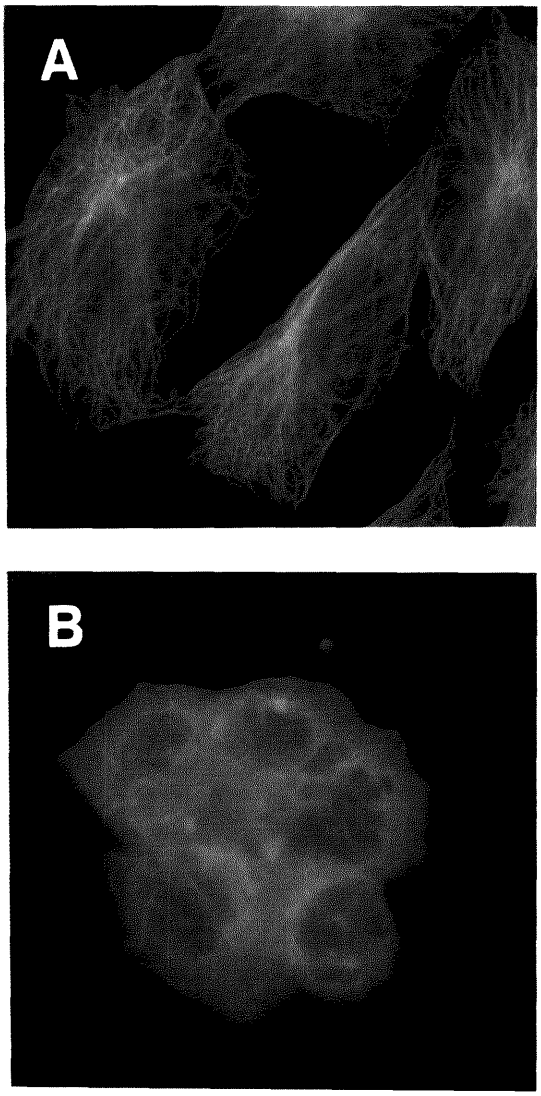

Fig. 4. Indirect Immunofluorescence Staining of Chinese Hamster V79 Cells with Anti-β-tubulin Antibody

Untreated cells (A) in interphase with extensive microtubule networks; cells treated for 3 h with (B) 100 μM DDS.

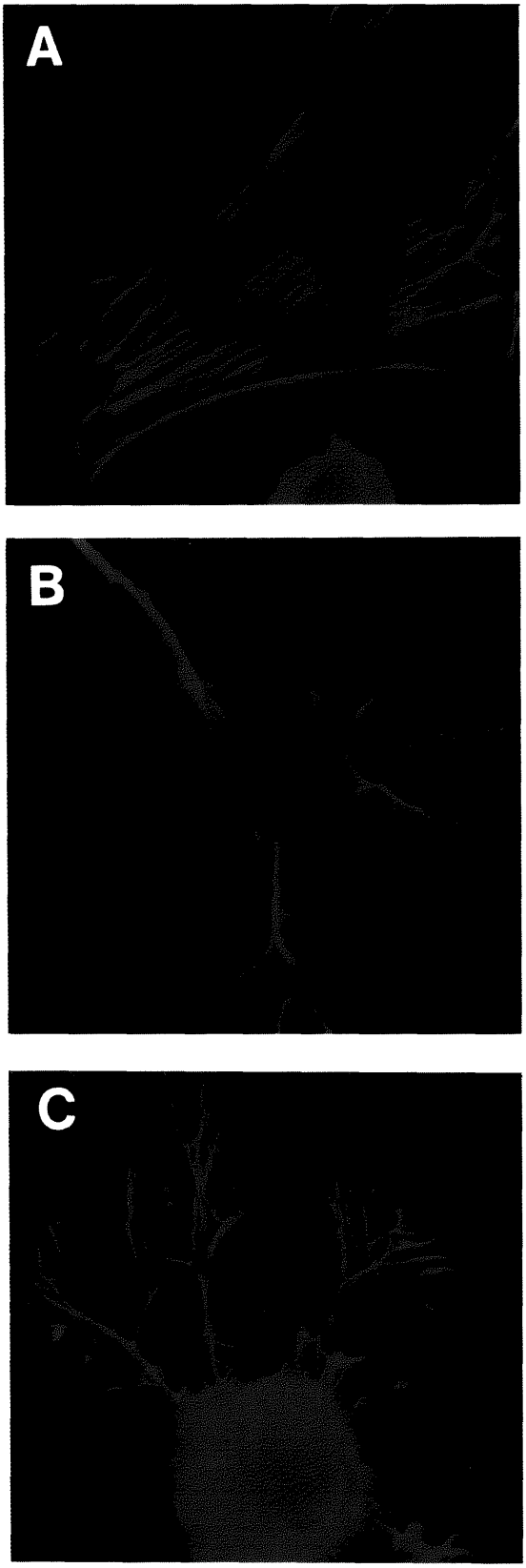

Fig. 5. Indirect Immunofluoresence Staining of Mouse BALB 3T3 Cells with Rhodamin-Conjugated Phalloidin

Untreated cells (A) in interphase with extensive actin stress fibers; cells treated for 3 h with (B) 100 μM DES and (C) 100 μM THS.

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was less effective than DDS.

Table I also shows the effects of DDS and THS on chromosome aberrations. Although 30 μM DDS induced chromosome aberrations, especially dicentrics and erosions, no chromosome aberration was observed under other treatment conditions.

Effects of DDS and THS on Cellular Microtubule Networks Though the aneuploidy frequencies of DDS and THS were very low, their effects on cytoplasmic microtubules were investigated. Interphase cells in unsynchronized cultures were examined by indirect immunofluorescence employing an anti-β-tubulin antibody.

The normal patterns of cytoplasmic microtubule disruption in the interphase cells are shown in Fig. 4A. Part of the cells treated with 100 μM DDS for 3 h changed in morphology to a round shape with accompanying microtubules, different from normal microtubules in the architecture. Short microtubules were observed around centromeres and they did not completely extend to the cell periphery (Fig. 4B). In a previous paper,[3] 30 μM DES completely disturbed the microtubule networks 1 h after treatment. One hundred μM DDS was required for complete disturbance during a 6 h treatment. On the other hand, 100 μM THS had no effect on cytoplasmic microtubule distribution during a 3 h treatment (data not shown).

Effect of DES, DDS and THS on Cellular Actin Networks Next, the effects of DES, DDS and THS on cytoplasmic actin stress fibers were investigated by indirect immunofluorescence employing a rhodamin-conjugated phalloidin.

The normal patterns of actin stress fiber distribution in the interphase cells are shown in Fig. 5A. Cells treated with 100 μM of DES and THS for 3 h changed in morphology to star shapes that were different from the disruptive pattern by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) previously reported[13] (Fig. 5B, C). On the other hand, 100 μM DDS had no effect on cytoplasmic actin networks during a 3 h treatment (data not shown).

In this paper, we examined the relation between microtubule disruptive activity and chemical structure using DDS and THS. The presence of the hydroxyl groups of DES is indispensable for inhibitory activity on microtubule assembly *in vitro*[2a] and microtubule disruptive activity in culture cells.[3] DDS showed half the inhibitory activity of DES *in vitro* and the disruptive activity of DDS was lower than DES in culture cells, indicating that the position of hydroxyl groups is also important for the activity. The result that THS had no activity either *in vitro* or in culture cells in spite of being substituted with four hydroxyl groups on the stilbene skeleton suggests that the presence of the ethyl groups is essential in this group of compounds.

In the previous paper,[3] the frequencies of aneuploidy in V79 cells treated with DES and its methyl ethers were consistent with the inhibitory activities on microtubule assembly *in vitro*. The induction of aneuploidy by DES and its methyl ethers could be explained by their inhibitory action on microtubule polymerization. In this study, 200 μM DDS showed inhibitory activity corresponding to 100 μM DES on microtubule assembly *in vitro*, and the frequency of aneuploidy by 30 μM DDS was similar to that by 15 μM DES. These results support the proposition by Wallin *et al.*[14] that the inhibition of microtubule assembly may be an inducer of aneuploidy.

It was found for the first time that DES had the disturbing activity on actin stress fiber distribution in BALB 3T3 cells. This may be concerned with the various activities of DES such as: synthetic estrogen,[15] carcinogen,[16] microtubule disruption,[1] biological activities (antifungal, antimicrobial, phytogrowth-inhibitory and ichthyotoxic activities, coronary vasodilator action on isolated guinea pig hearts),[5a] inhibition of transcription,[17] *etc.* THS, which did not have the distrubing activity on microtubule distribution in V79 cells, showed this activity similar to DES on actin stress fiber distribution in BALB 3T3 cells. On the other hand, although DDS showed the disturbing activity on microtubules, it had no activity on actin stress fibers.

Some microtubule associated proteins can cross-link actin filaments *in vitro*,[18] and interact with actin filaments *in vivo*.[19] Danowski reported that the disruption of actin stress fibers by 12-*O*-tetradecanoylphorbol 13-acetate is reversed by the microtubule poisons, colcemid, vinblastine and nocodazole. He considered the possible explanation that microtubules modulate, in an inhibitory fashion, the state of organization of cytoplasmic actin.[12] Furthermore, Okuhara *et al.* reported that MAP kinesin associates with actin stress fibers following experimental disruption of microtubules.[20]

The results in this study using the microtubule disruptive agent, DES, however, were inconsistent with the proposition by Danowski[12] that the release of MAPs from the microtubules by microtubule poisons stimulates the actin organization into stress fibers. This suggests that DES has various activities in the culture cells, and it is valuable that the DES related compounds, DDS and THS, showed different disturbing activities on microtubules and actin stress fibers.

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