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A Comparative In Vitro Study: Cytotoxicity Effect of Curcumin Vs Biodegradable Nanoparticles/PVA Against HNO97 And Effect of Curcumin on Cell Cycle Phases

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ABSTRACT

Purpose: This study evaluated that curcumin and biodegradable nanoparticles with PVA as capping material, in terms of cytotoxicity followed by cell cycle analysis against the HNO97 cell line (tongue carcinoma). **Material and methods:** 5mg of pure curcumin, 7.5 mg of Nano curcumin formulation, and SRB for IC50 done on five concentrations. A flow cytometry test was carried on to evaluate the effect of curcumin on the HNO97 cell line. **Results:** This study postulated that curcumin had the highest cytotoxicity effect against cell line (HNO97) with IC50=1.59 μg/ml in comparison to curcumin nanoparticles/PVA showed low cytotoxicity owing to the pH (7.4) and surface charges of PVA. Also, the cell cycle test for curcumin showed that the percentage of cells in G2/M phase was significantly increased (p=0.003). **Conclusion:** The study showed that curcumin has a powerful anticancer effect against in vitro tongue carcinoma. Generally, these results reported that curcumin can in vitro inhibit cancer cells.

KEYWORDS

HNO97, PVA, Curcumin, Cytotoxicity, Flow cytometry

INTRODUCTION

According to the International Classification of Diseases coding scheme and WHO, oral cancer (OC) is the 8th most frequent type of cancer in the world among males and 14th among females [(1)]. Elder people are most affected between 50 and 70 years old, though, recent articles submit that OC happens in infants as early as 10 years old, without any known etiology [(2)]. The causes of OC are multiple; the most common etiology is smoking and alcohol abuse [(3)]. Oral squamous cell carcinomas approximately represent (84-97%) of the OCs. The regimen of oral squamous cell carcinomas (OSCCs) involves surgery, chemotherapy, and radiotherapy. Regrettably, about 50% of the cases cannot accomplish these regimens because of toxic effects [(4)].

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Oral squamous cell carcinomas are considered all over the world the most common cancer. The survival rate is weak, in spite of, the available regimens. Adding mediators to make chemotherapeutics safer and more effective [5].

Curcumin is a natural alkaloid product applicable for many centuries for many diseases involving cancer and public Indian flavor that has revealed anticarcinogenic behaviors [4].

Phytochemical curcumin has been massively explored for its beneficial uses through in vitro and in vivo studies. As it hits many cancers and is suggested to sensitize cancer drugs in multidrug-resistant cancers (MDR) [6]. It has been revealed to own biological activity against many bands of physiological conditions involving antioxidant, anti-diabetic, chemo-protective, and against cancer cells revealed anti-proliferative activity [6].

Besides, the anti-proliferative behaviors of Curcumin have been generally calculated against many forms of cancers because it has the ability to hit many routes in cancer. Even though Curcumin displayed a potent anticancer action, its clinical application is limited attributed to its earlier metabolism and little water solubility [6].

Several studies have revealed the beneficial role of Curcumin in many types of cancer, involving OC. Epidermal growth factor receptor (EGF) and its downstreaming signals route act as a significant role in OSCC pathogenesis and are reduced by Curcumin. Curcumin hearsays reduce the proliferation of the cells and apoptosis in OSCC [7].

Nanotechnology by using Nano formulations of curcumin including liposomes, biodegradable nanoparticles, phospholipids complexes, and micelles have defined[8]. The capping materials of curcumin into polymeric nanoparticles such as poly lactic-co-glycolic acid (PLGA), polyvinyl pyrrolidone (PVP), and polyvinyl alcohol (PVA). The probable efficiency of Nano curcumin and its effects on OSCC cell lines has been discovered sufficiently [8].

The purpose of this study was to assess Curcumin and biodegradable nanoparticle with PVA as a capping material, in terms of cytotoxicity followed by cell cycle analysis against the HNO97 cell line (tongue carcinoma).

MATERIAL AND METHODS

BUC-Institution Ethical Committee for Faculty of Oral and Dental Medicine for Badr University in Cairo approval was obtained (BUC-IACUC-221130-12). Sample size is calculated to be 5 mg of pure curcumin and 7.5 mg of Nano curcumin formulation [9].

1. Cell line culture:

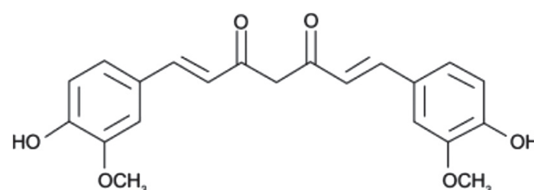
HNO97: SCC of the tongue was acquired from Nawah Scientific laboratory, (El-Mokatam, and Cairo, Egypt). Keeping cells in the media of DMEM complemented with streptomycin (100 mg/mL), penicillin (100 U/mL), the Heat-Inactivated Fetal Bovine Serum (FBS) in humidified (10%), 5% (v/v) CO_2 atmosphere at 37 °C.

2. Curcumin preparation:

Curcumin ≥ 98% with molecular weight =368.38, Empirical Formula $C_{21}H_{20}O_6$, CAS Number 458-37-7, Catalog Number HIKA20091, Lot Number 22A1, plant source from Egypt.

Product results:

Result	Specification	Test Value
Colour	Yellow to orange powder	Yellow to orange powder
Form	Solid	Solid
Purity (HPLC)	≥ 98 %	99 %
MS	Authentic	Authentic



3. Biodegradable Nanoparticles:

Curcumin Nanoparticles, brand (NT-CNR-NPS) with Code 4020 by Nanotech Egypt for Photo-Electronics Supplier. Optical properties: UV-Vis absorption spectra were obtained on an Ocean Optics USB2000+VIS-NIR Fiber optics spectrophotometer.

Size and shape: TEM was performed on JEOL JEM-2100 high-resolution transmission electron microscope (TEM) at an accelerating voltage of 200 kV, correspondingly. Characterizations were yellowish brown in color, powder form, with polyvinyl alcohol (PVA) capping material, suspension in water, colloidal in Ethanol, optical prop. (Abs.): λ max =425 nm, 50± 5.5 nm in size, and spherical like-shape. Sample (s) Information: 5 mg of pure curcumin, 7.5 mg of Nano curcumin formulation and SRB for IC50 done on 5 concentrations.

4. Cytotoxicity test:

The viability of cells was evaluated by the sulphorhodamine B (SRB) test. Enumerative of 100µL cell suspension ($5x10^3$ cells) placed in microtiter plates and raised for 24h in complete media. Treating cells are put with another enumerative of 100µL media comprising medications at different concentrations. After medication exposures for 72 hrs, fixation of cells by changing media with 150 microlitres of TCA (10%) and raised for 60 minutes at 4°C. The liquid of TCA was eliminated, and cells was cleaned for 5 periods with purified water. Enumerative of 70µL SRB liquid (0.4%w/v) were put and implanted at room temperature for 10 min in a dark area. Cleaning microtiter plates with 1% of acetic acid for 3 periods and permitted to be dehydrated overnight. After that, dissolving protein-bound SRB by putting 150µL of TRIS (10mM); using a BMGLABTECH®- FLUO star Omega microtiter plate reader (Ortenberg, Germany) to evaluate the absorbance at 540 nm. [(9)]

5. Assay for Cell Cycle Distribution:

Cells (105 cells) were collected by trypsinization and cleaned two times with ice-cold PBS (pH 7.4), after treatment with test compounds for a day or 2 days and paclitaxel (1µM) as a positive control for 24h. Resuspension of cells was done in two millilitres of 60% ice-cold ethanol, then, implanted for a day at 4ºC for fixation. The fixed cells were immersed two times with PBS after that resuspended in 1 mL of PBS carrying 10 µg/mL propidium iodide (PI) and 50 µg/mL RNAse A. Placement in a dark place at 37 C after 20 minutes, cells were examined for DNA by means of flow cytometry test via FL2 (λex/ em 535/617 nm) signal detector (ACEA Novocyte™ flow cytometer, ACEA Inc., CA, San Diego, USA). For each model, 12,000 trials are obtained. The distribution of the cycle of the cell was determined using ACEA NovoExpress™ software (ACEA Biosciences In company, San Diego, USA). [(10, 11)]

Statistical analysis:

A statistical study was done by application R version 4.0.4. One-Way ANOVA was used to show if there is significance among groups. Tukey multiple pairwise comparison were used to compare every two groups. The outcomes are viewed as mean with the standard of error (SE) with less than 0.05 (P-Value) measured to be significant.

Data were examined using the analytical software package (IBM-SPSS) version 22. According to the Kolmogorov-Smirnov test, the data were normally distributed. One ANOVA was applied to evaluate phases effect on the % distribution of the cells in each experimental group. Test of Duncan was indicated to study the resemblance over the different phases in each group. Performance of independent t-test to explain the differences in a statistical study, compared to the control. Data were displayed as mean ± standard error of mean.

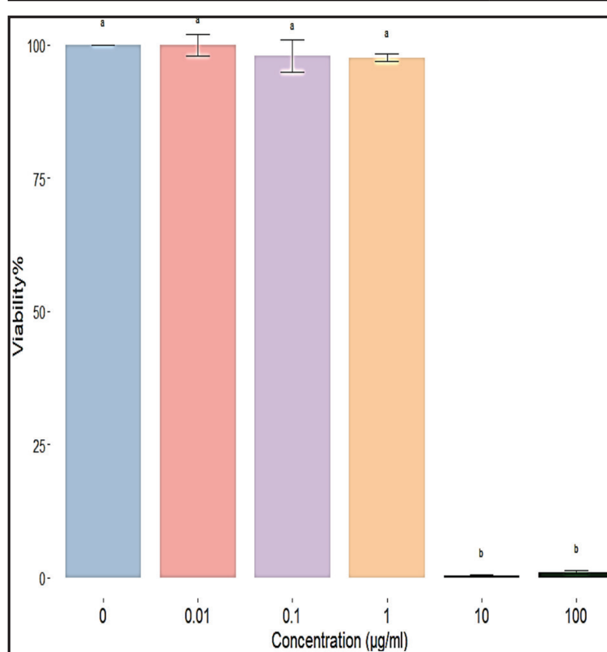
RESULTS

1. Cytotoxicity test results:

As shown in (table 1) and (figure 1), curcumin had no effect on the cell's viability when its concentration below or equal to 1(μg/ml) cells viability were (99, 98 and 98%) at (0.01, 0.1, 1 μg/ml) conc. of curcumin, respectively. However, cell viability % were decreased below 1% at the largest two concentrations 10 (μg/ml) and 100 (μg/ml) by (0.34 and 0.99%), respectively with p-Value <0.000. On the other hand, Nano-Curcumin has a poor result as shown in (table 2) where cell viability decreased from 100% in the control group to 97% in the highest concentration of 100(μg/ml) although this decrement was not significant (Figure 2). As Curcumin with IC50 equal 1.59 μg/ml.

Table 1 and 2: *Viability of HNO 97 cell line among different curcumin and Nano-curcumin concentrations:*

Nano-Curcumin Concentration (µg/ml)	Viability% (Mean ± STD)	p- Value	Curcumin Concentration (µg/ml)	Viability% (Mean ± STD)	p-Value
0	100±0.0		0	100±0.0	
0.01	101±0.78	0.156	0.01	99±1.6	<0.000
0.1	100±1.76		0.1	98±2.5	
1	98±1.02		1	98±0.6	
10	97±2.8		10	0.34±0.16b	
100	97±2.0		100	0.99±0.26b	



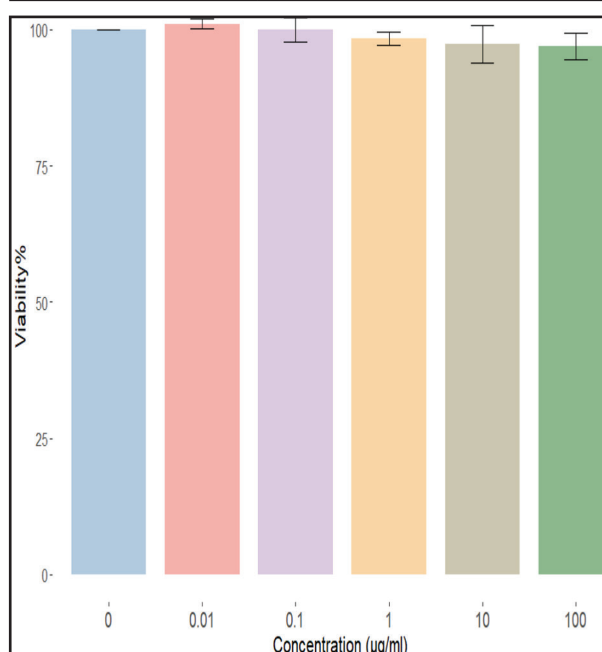


Figure (1 & 2) Bar charts comparing the mean of cell viability among different Curcumin and Nano- Curcumin concentrations and the error columns reveals the error of standard of each set. Different letters mean significant difference between groups according to Tukey multiple pairwise-comparisons.

2. Test of flow cytometry:

The cell distribution percentage over stages of the cell cycle in the control and curcumin-treated groups are displayed (Table 3 & figure 4). Among the controlled group and curcumin-exposed one, the highest percentage of cells were found in the phase of G1, followed by G2-phase, then the S-phase, and finally the sub-G1. However, as compared to the controls, the percentage of cells in the G1 phase was significantly reduced (p=0.006), whereas the percentage of the cells in the phase of G2/M was elevated significantly (p=0.003), indicating the anticancer effect of curcumin.

Table (3) *Show curcumin effects on the arrest of the cell cycle in HNO 97. The number of cells when treated with curcumin in Sub G1, G0/G1, S, and G2/M of the cell cycle.*

Group	Stages of cell cycle				Effect of phases
	Sub G1	G1/G0	S	G2/M	
Control	1.15 ± 0.06^{A}	60.15 ± 0.60^{D}	22.69 ± 0.48^{B}	25.60 ± 0.89^{C}	$F_{3,8}$= 1716, P=0.000
Curcumin	$3.61 \pm 0.06^{*A}$	$51.21 \pm 1.56^{*D}$	20.50 ± 0.98^{B}	$32.54 \pm 0.62^{*C}$	$F_{3,8}$= 426, P=0.000
Effect of treatment	P=0.000	P=0.006	P=0.115	P=0.003	

In the similar row, means listed with the same capital letters showed insignificant differences with p>0.05, while others considered with different ones revealed a significant difference with P<0.05.

**: significant differences as compared to the controls.*

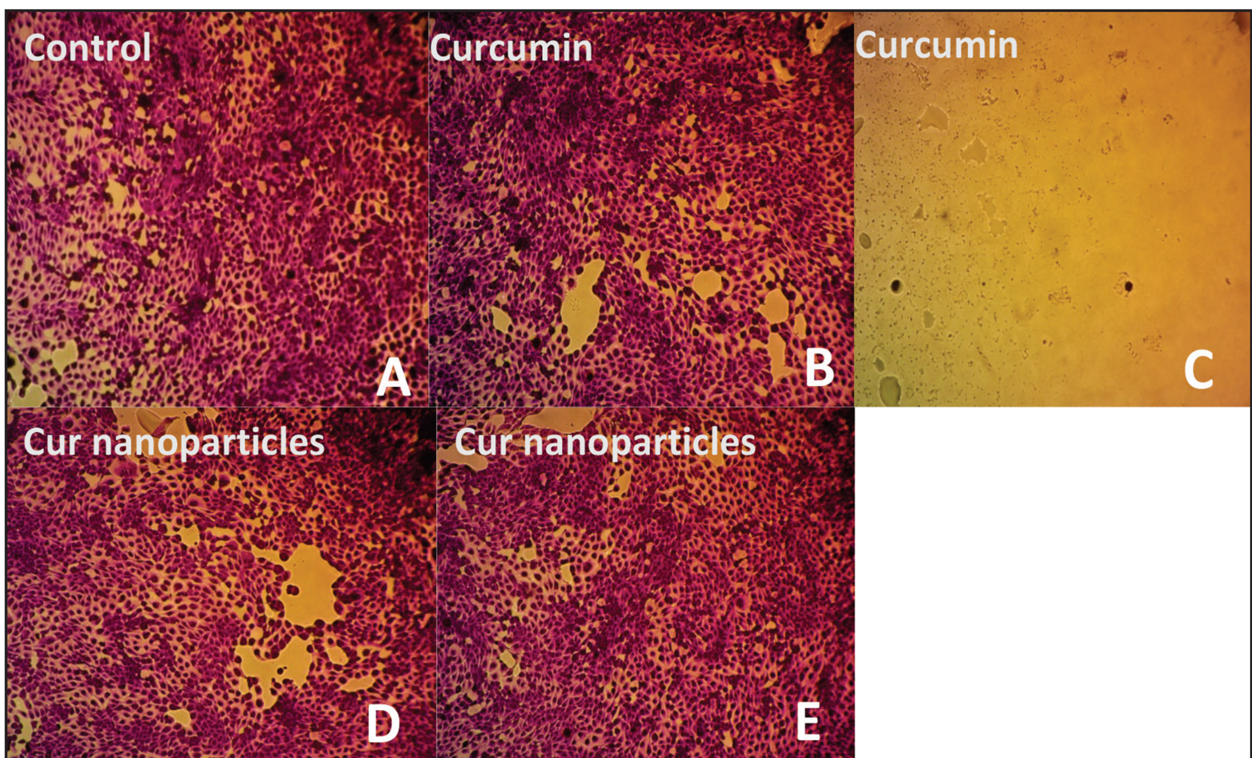


Figure (3) Light microscopy at 100× magnification Fig. (A) Control, fig. (B) effect of Curcumin at 1 μg/mL conc. on colony formation HNO97, fig (C) curcumin at 100 μg/mL conc., fig. (D and E) showing effect of cur. Nanoparticle at 1 and 100 μg/mL conc., respectively on colony formation HNO97. Illustrate the effect of Curcumin vs Nano curcumin on both concentration (1 and 100 μg/mL) conc., respectively on HNO97. Given that curcumin had an effective cytotoxicity on cells as shown in fig. (c).

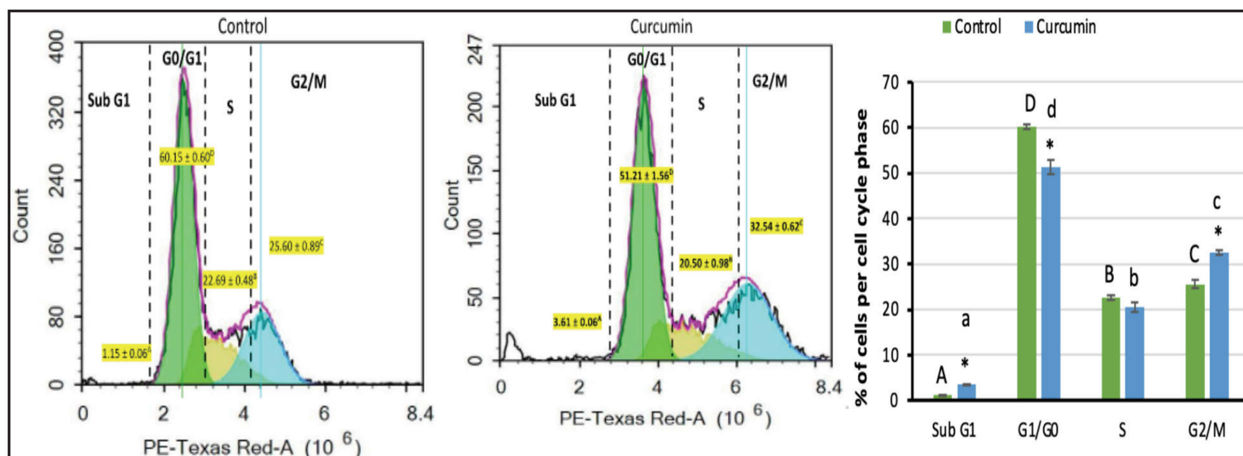


Figure (4) Effects of curcumin on cell cycle arrest in HNO 97 cell line. Population histogram for the control and curcumin-treated samples. Generated with Novo Express software. The sub-G1 population is indicated in white. G0/G1 population is in green, S phase population is in yellow, and the G2/M phase population is in cyan (Left side). The cells percentage in Sub G1, G0/G1, S, and G2/M phases in the cell cycle when exposed to curcumin (Right side). Among the cell cycle phases of control (capital letters) and curcumin-treated (small letters) groups, phases with different letters show a significant difference ($p<0.05$).

*: significant differences as compared to the controls.

DISCUSSION

According to this study, curcumin showed a significant effect on cell viability at 10 and 100 (μg/ml) concentrations with IC50: 1.59 μg/mL after 72 hours. In accordance with the study presented that curcumin hindered cell proliferation significantly at 5 μM concentration and the amount of inhibition touched 50% nearly at 35 µM [12].

The results for curcumin nanoparticles aren't affected the cell viability as the percentage of cell viability starts to decrease from 100% to 97% at (10 and 100 μg/ml) as conducted in a study that cytotoxicity in vitro assay of the leached curcumin revealed constant antiproliferation impact for 27% inhibition against cell line HEPG2 which prolonged up to 25 days and also, other study indicated that curcumin kills tumor cells selectively [13].

May be due to the neutral media and the negative charge of the capping material PVA. These findings are in accordance with a study conducted that the leaching rate of curcumin from studied curcumin /PVA polymers was elevated in basic, acidic, and inhibited in the neutral one. Along with the following order: alkaline pH > acidic pH >neutral pH the leaching rate was decreased. [13] Also, there was a study postulated that these behaviors were related to increasing the solubility of curcumin in both acidic and alkaline media but less solubility in distilled water. [14] A study conducted that the more the diffusion coefficient is, the higher the release rate will be. Consequently, the more curcumin solubility enhanced its diffusion coefficient and the release rate is elevated. Therefore, the diffusion was attributed to the aqueous media type and characteristics of the material composition [15].

Alterations in the zeta potential of curcumin nanoparticles attributed to polymers type used for coating [16]. A study was done to prove that PVA-NPs-Cur were charged negatively due to the hydroxyl group. Nanoparticles charged positively showed higher cytotoxicity effects in comparison to negative and neutral [17].

Many findings have reported that the nanoparticles with positive charges showed more toxicity

effects to cells. For example, a study assessed that positive monolayer layered gold NPs were more cytotoxic on cells of Cos-1 in comparison to negatively layered gold NPs.[18] Another study proved that poly (ethylenimine) covered iron oxide NPS (+55.6 mV) positively charged donated to more cytotoxic effect related to endocytosis elevated the uptake of the NPs. In comparison, additional covering of poly ethylene glycol- polyvinyl alcohol (PEG-PVA) altered the surface charges to more negative values, thus lowering its cytotoxicity on the cells. [19]

A study showed that the prepared magnetic nanoparticles MNPs (MgFe2O4 ferrite) were accomplished by DOX encapsulation. These MNPs were successfully put to work with the three polymers; chitosan (CHI), polyvinyl alcohol (PVA), and polyethylene glycol (PEG), with CHI- MNPs revealing the highest DOX loading but PVA- MNPs had the lowest DOX encapsulation competence. Also, The DOX-CHI-MNPs had predisposed to superior anticancer activity, in comparison to DOX-PVA-MNPs. This study also, assured that the rate of DOX release was pH-dependent, increasing with a decrease in PH 4.5, 6.5, and 7.4, respectively. [20]

The cytometry flow test was indicated for revealing the effect of curcumin on cycles of the cell distribution of HNO97 cells using PI staining after 24 h of treatment.

One of the main features of curcumin is cell cycle arrest in malignant cells, which cause a change in cells number at different stages. The four phases of the cell cycle is: G1, S, G2, and M [21]. In the current study attributed to flow cytometry analysis, in G2/M is increased and in G1-phase the cells percentage is reduced in comparison to the control group. In acceptance with other studies [22] reported that curcumin and DMC encourage G2/M phase arrest in oral SCC-25 and human glioma U87 MG cells, correspondingly. [23] Curcuminoids elevated G2/M phase percentage cell populations from 2.6±0.2 in the untreated cells to 8.6±0.95 in the treated cells.

CONCLUSIONS

Curcumin has a powerful cytotoxic effect against tongue SCC and show a critical role in cell cycle phases. The investigated biodegradable nanoparticles/PVA has an effect on curcumin release related to pH and surface charges due to the hydroxyl group in which more researches are essential at these points to clarify these factors.

RECOMMENDATIONS

Further studies are needed to confirm the results of the present study for a better understanding of the action of biodegradable nanoparticles/PVA on tongue carcinoma.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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