# SULPHAMOYLATED ESTRADIOL ANALOGUE INDUCES ANTIPROLIFERATIVE ACTIVITY AND APOPTOSIS IN BREAST CELL LINES

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**Abstract**

Research regarding possible anticancer agents has shown that 2-methoxyestradiol exerts *in vitro* and *in vivo* antiproliferative activity in an estrogen receptor independent manner. Due to limited biological accessibility and rapid metabolic degradation several new analogues were developed in recent years. This study investigated the *in vitro* effects of a novel *in silico*-designed compound (compound 16 (C16)) in an estrogen receptor positive breast adenocarcinoma epithelial cell line (MCF-7), an estrogen receptor negative breast epithelial cell line (MDA-MB-231) and a non-tumorigenic breast cell line (MCF-12A). Light microscopy revealed decreased cell density, cells blocked in metaphase and the presence of apoptotic characteristics after C16 exposure for 24 h in all three cell lines. Polarization-optical transmitted light differential interference contrast designated the presence of several rounded cells and decreased cell density. The xCELLigence real-time label-independent approach revealed that C16 exerted antiproliferative activity. Significant inhibition of cell growth was demonstrated with 0.2µM after 24 h exposure in all three cell lines. However, the non-tumorigenic MCF-12A cell line recovered exceedingly after 48 h when compared to the tumorigenic cell lines. This short communication indicates that C16 acts as an antiproliferative agent, possesses antimitotic activity and induces apoptosis *in vitro*, warranting further investigations.

Keywords: C16, Cancer, Proliferation, MCF-7, MCF-12A, MDA-MB-231, Metaphase, Apoptosis, Xcelligence, Tumorigenic

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Abbreviations: 2-Methoxyestradiol (2ME); Compound 16 (C16); Hours (h); Minutes (min)

**INTRODUCTION**

2-Methoxyestradiol (2ME) is a 17-beta estradiol derivative that exerts antiproliferative activity and destroys the tubulin structure in an estrogen receptor independent manner. 2ME failed to advance to United States Food and Drug Administration approval because of its low efficacy [1, 2, 3, 4]. Due to rapid metabolic degradation and limited bioavailability, several promising analogues were developed in recent years [2].

2-Methoxyestradiol-bis-sulphamate is a bis-sulphamoylated analogue with antiproliferating activity that induces apoptosis in several cell lines including estrogen receptor positive breast adenocarcinoma cells (MCF-7), prostate cancer cells (PC-3), human umbilical vein endothelial cells (HUVEC) and the human breast adenocarcinoma CAL51 cell line [2, 5, 6, 7, 8]. 2-Methoxyestradiol-bis-sulphamate exposure resulted in a $G_2$/M block in cell lines including the estrogen receptor positive human breast adenocarcinoma cell line (MCF-7), the drug resistant human adenocarcinoma cell line (MCF-7 DOX40) and the highly tumorigenic breast carcinoma MDA-MB-231 cell line [9]. Another analogue, 2-methoxyestra-1,3,5(10)16-tetraene-3-carboxamide (also known as ENMD-1198, C24, 883, or ENMD-0998) exerts antiproliferative- and antiangiogenic activity in the MCF-7 cell line and the colon cancer cell line (HCT 116) [10, 11, 12]. Sulphamoylated analogues including, methylcoumarin-sulphamate and 2-methoxyestradiol-sulphamate exert steroid sulphatase inhibitory activity with methylcoumarin-sulphamate possessing additional weak aromatase activity and are currently being evaluated in Phase I clinical trials for postmenopausal women with metastatic breast cancer [13, 14, 15].

This preliminary study focuses on the differential effects of a novel sulphamoylated 2ME compound (compound 16 (C16)) that was *in silico*-designed in our laboratory based on the anticancer activity of other above-mentioned sulphamoylated compounds (Fig. 1). Docking studies revealed that 2-ethyl-3-Osulphamoyl-estra-1,3,5(10)16-tetraene (Compound 12), 2- ethyl-3-O-sulphamoyl-estra-1,3,5(10),15-tetraen-3-ol-17-one (Compound 9) and 2-ethyl-17-(1'-methylene)estra-1,3,5(10)-trien-3-Osulphamate possessed superior carbonic anhydrase IX / carbonic anhydrase II ratio when compared to other synthesised molecules and its parental molecule (2ME). Furthermore, hydrophobic interactions between Compound 12 and ala250.B, leu242.B, leu248.B, leu252.B, leu255.B, lys352.B, and val318.B were demonstrated [16]. These studies showed that the *in silico*-designed compounds are more potent than 2ME2, and they present with 50% growth inhibitory ($GI_{50}$) in the nanomolar range [16]. *In vitro* effects of C16 were investigated on cell proliferation, morphology and possible cell death induction in an estrogen receptor breast adenocarcinoma cell line, an estrogen receptor negative metastatic cell line and a non-tumorigenic breast epithelial cell line.

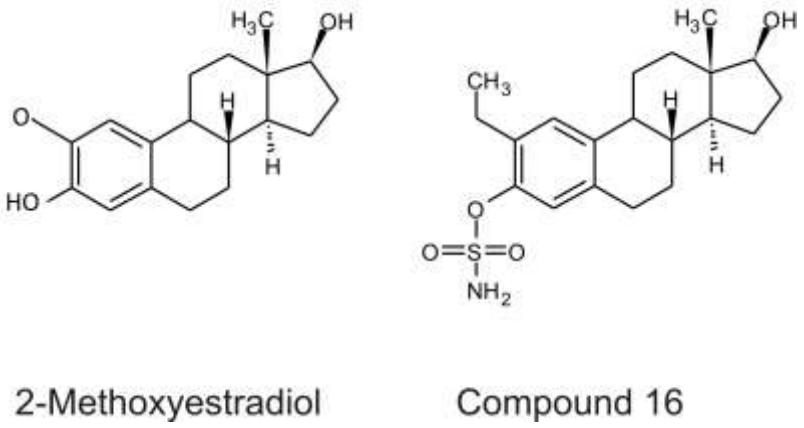


Fig. 1: C16 is a sulphamoylated analogue of 2ME. Structural differences between 2ME and C16 are summarized.

## METHODS AND MATERIALS

### Materials

*Cell line*

MCF-7 is an estrogen receptor positive tumorigenic adherent breast epithelial cell line derived from metastatic sites in adenocarcinoma. MCF-7 cells were supplied by Highveld Biological (Pty) Ltd. (Sandringham, South Africa). MDA-MB-231 is an estrogen receptor negative breast adenocarcinoma cell line supplied by Microsep (Pty) Ltd, Johannesburg (South Africa). MCF-12A is a non-tumorigenic transformed adherent human breast epithelial cell line. These cells are produced by long-term cultures and form domes in confluent cultures. The MCF-12A cells were a gift from Professor Parker (Department of Medical Biochemistry, University of Cape Town, South Africa).

*Reagents*

All required reagents of cell culture analytical grade were purchased from Sigma (St. Louis, United States of America) unless otherwise specified. Heat-inactivated fetal calf serum (FCS), sterile cell culture flasks and plates were purchased from Sterilab Services (Kempton Park, Johannesburg, South Africa). Penicillin, streptomycin and fungizone were obtained from Highveld Biological (Pty) Ltd. (Sandringham, South Africa). The sulphamoylated analogue (C16) of 2-methoxyestradiol was synthesized by iThemba Pharmaceuticals (Pty) Ltd. (Modderfontein, Gauteng, South Africa) since this compound was *in silico*-designed in our laboratory and therefore commercially unavailable [13]. A stock solution of C16 dissolved in dimethyl sulphoxide (DMSO) was prepared with a concentration of 10mM and was stored at 4°C. The vehicle control sample composed of DMSO and growth medium where the DMSO content of the final dilutions never exceeded 0.05% (v/v).

### Methods

*Cell culture*

Cells were grown and maintained in 25cm$^2$ tissue culture flasks in a humidified atmosphere at 37°C and 5% $CO_2$. MCF-7- and MDA-MB-231 cells were cultured in Dulbecco's minimum essential medium eagle (DMEM) and supplemented with 10% heat-inactivated FCS (56°C, 30min), 100U/ml penicillin G, 100µg/ml streptomycin and fungizone (250µg/l) [9]. MCF-12A maintenance medium consisted of a 1:1 mixture of DMEM and Ham's-F12 medium, 20ng/ml epidermal growth factor, 100ng/ml cholera toxin, 10µg/ml insulin and 500ng/ml hydrocortisone, supplemented with 10% heat-inactivated FCS (56°C, 30min), 100U/ml penicillin G, 100µg/ml streptomycin and fungizone (250µg/l) [2].

*Light microscopy (Haematoxylin and eosin staining)*

Investigation regarding the *in vitro* influence of C16 on cell morphology was conducted using the haematoxylin and eosin staining method. Cells were seeded on sterile coverslips in 6-well plates at a density of 500 000 cells per well and incubated overnight. Afterwards cells were exposed to 0.2 µM C16 for 24 h since previous studies have shown that the sulphamoylated compounds exert antiproliferative and apoptotic activity at 0.2µm (24 h) [16]. Haematoxylin and eosin staining method was conducted according to Visagie *et al*. (2010) [2]. Coverslips were mounted on microscope slides with resin and left to dry. Photos were taken utilizing a Zeiss Axiovert MRc microscope (Zeiss, Oberkochen, Germany). In addition, haematoxylin- and eosin-stained cells were used to determine mitotic indices. Quantitative data for the mitotic indices was acquired by counting 1,000 cells on each slide of the biological replicates and expressing the data as the percentage of cells in each phase of mitosis (prophase, metaphase, anaphase and telophase), cells in interphase and cell demonstrating hallmarks of apoptosis. This haematoxylin and eosin staining yielded both qualitative and quantitative information.

*Polarization-optical transmitted light differential interference contrast*

Polarization-optical transmitted light differential interference contrast (PlasDIC) is a method to view cell morphology and was conducted according to Visagie *et al.* (2010) and Visagie *et al.* (2011) [2, 9]. Images were obtained before and after exposure using the Axiovert 40 CFL microscope (Carl Zeiss, Goettingen, Germany).

*xCELLigence monitoring*

The xCELLigence system is a novel approach developed by Roche Applied Science (Penzberg, Germany) to investigate cell growth, adhesion and morphology in real time using a label-independent manner and was employed to confirm whether the dosage exerts optimal antiproliferative activity [17]. This system measures electrical impedance across the micro-electrodes integrated on the bottom of tissue culture 96-well plates therefore allowing real-time and continuous cellular analysis as cells attach and proliferate. The change in impedance is expressed as the cell index. The cell index is an indication of cell number, cellular attachment and morphology. Cells were seeded at a density of 5000 cells per well, placed for 30 min on a rotator plate and was subsequently placed in the xCELLigence system that was linked to the incubator in a humidified atmosphere at 37°C and 5% $CO_2$ [17].

## STATISTICAL ANALYSIS

Qualitative data were supplied by PlasDIC, light microscopy (haematoxylin and eosin staining) and fluorescent microscopy. Quantitative information was gained by means of time- and dose dependent studies utilizing the real time xCELLigence system. Data were obtained from three independent experiments with 6 technical repeats each. Data were statistically analysed for significance using the analysis of variance (ANOVA)-single factor model followed by a two-tailed Student's *t*-test. *P*-values $< 0.05$ were regarded as statistically significant.

## RESULTS

### Light Microscopy

Light microscopy of C16 exposure to MCF-7, MCF-12A and MDA-MB-231 cells were performed to investigate *in vitro* effects on morphology (Fig. 2). C16 exposure resulted in compromised cell density in all three cell lines when compared to vehicle-treated cells. Several apoptotic hallmarks (apoptotic bodies, shrunken cells and cell debris) and cells blocked in metaphase were observed in treated MCF-7 cells (D), treated MDA-MB-231 cells (E) and treated MCF-12A cells (F). MDA-MB-231 cells were prominently affected by C16 compared to the other cell lines. Mitotic indices (Table 1) revealed that a significant percentage of cells present in metaphase and demonstrated features of apoptosis after exposure to C16 in all three cell lines when compared to vehicle-treated cells.

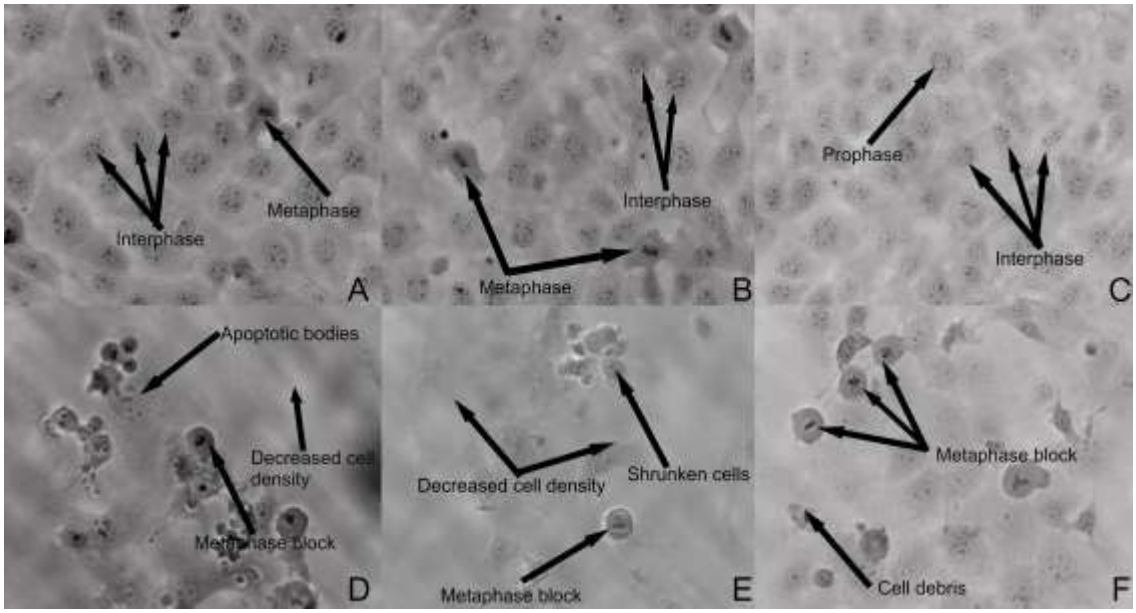


Fig. 2: Light microscopy of haematoxylin and eosin staining regarding MCF-7 vehicle-treated cells (A), MDA-MB-231 vehicle-treated cells (B), MCF-12A vehicle-treated cells (C), C16-treated MCF-7 cells (D), C16-treated MDA-MB-231 cells (E) and C16-treated MCF-12A cells (F). All cell lines treated with C16 revealed decreased cell density and an increase number of cells present in metaphase

when compared to vehicle-treated cells. In addition, apoptotic bodies, shrunken cells and cell debris were observed. All micrographs were taken at 40 X magnification.

Tab. 1: Percentages of cells in mitosis, interphase and cells featuring characteristics of apoptosis

| | Interphase | Prophase | Metaphase | Anaphase | Telophase | Apoptotic cells |
|---|---|---|---|---|---|---|
| **MCF-7** | | | | | | |
| Vehicle-treated cells | 93.5% | 3.6% | 1.2% | 0.9% | 0.8% | 0.0% |
| C16-treated cells | 59.4% | 1.1% | 28.8 % | 0.1% | 0.2% | 10.4% |
| **MDA-MB-231** | | | | | | |
| Vehicle-treated cells | 93.3% | 3.3% | 1.6% | 1.0% | 0.8% | 0.0% |
| C16-treated cells | 53.9% | 0.9% | 32.5% | 0.2% | 0.1% | 12.4% |
| **MCF-12A** | | | | | | |
| Vehicle-treated cells | 94.5% | 3.2% | 1.2% | 0.6% | 0.5% | 0.0% |
| C16-treated cells | 56.7% | 1.9% | 30.4% | 0.5% | 0.2% | 10.3% |

**Polarization-optical transmitted light differential interference contrast**

Polarization-optical transmitted light differential interference contrast (PlasDIC) indicated the induction of apoptosis in all MCF-7, MDA-MB-231 and non-tumorigenic MCF-12A cells (Fig. 3). Cells were exposed for 24 h to C16 and demonstrated decreased cell density with C16-treated cells appearing rounded when compared to the vehicle-treated cells. In addition, treated cells demonstrated shrunken cells, cell debris and apoptotic bodies which are all charateristics of apoptosis.

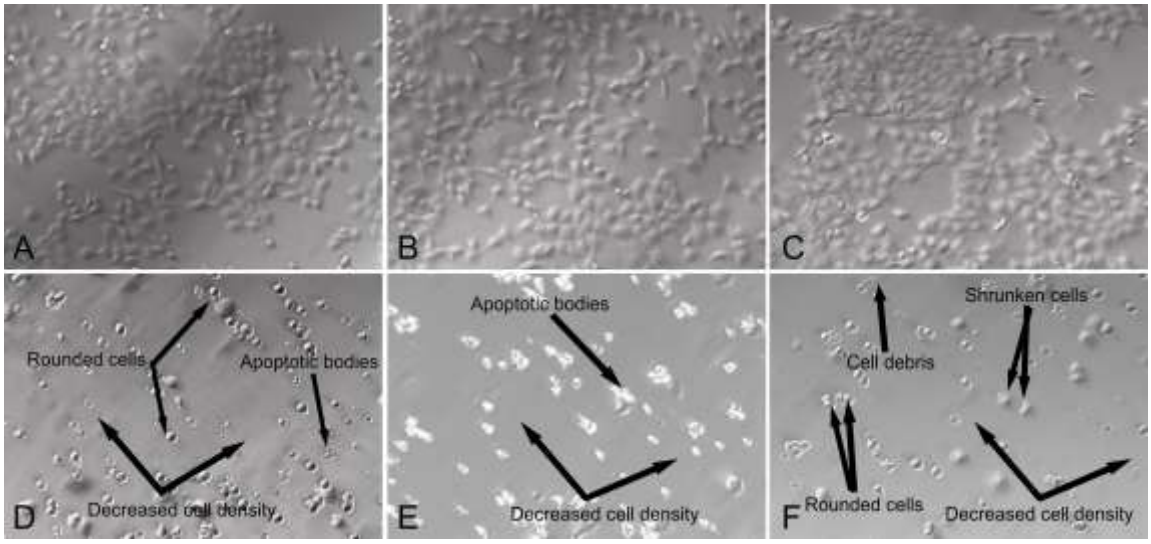


Fig. 3: PlasDIC micrographs of vehicle-treated MCF-7 cells (A), MDA-MB-231 cells (B), MCF-12A cells (C) C16-treated MCF-7 cells (D), C16-treated MDA-MB-231 cells (E) and C16-treated MCF-12A cells (F). Cells were exposed to 0.2µM of C16 for 24 h. Vehicle-treated cells showed no abnormal morphology. C16-treated cells revealed decreased cell density, rounded cells, apoptotic bodies and cell debris.

**xCELLigence system**

This novel real time label-independent approach measured cell adhesion and cell proliferation of C16 exposure to MCF-7, MDA-MB-231 and MCF-12A cells (Fig.4A, 4B and 4C). Cell proliferation was significantly inhibited in all cell lines (0.2-1µM) decreasing the cell index in the MCF-7 and MDA-MB-231 cell line. The MCF-12A cell line recovered after 48 h of exposure.

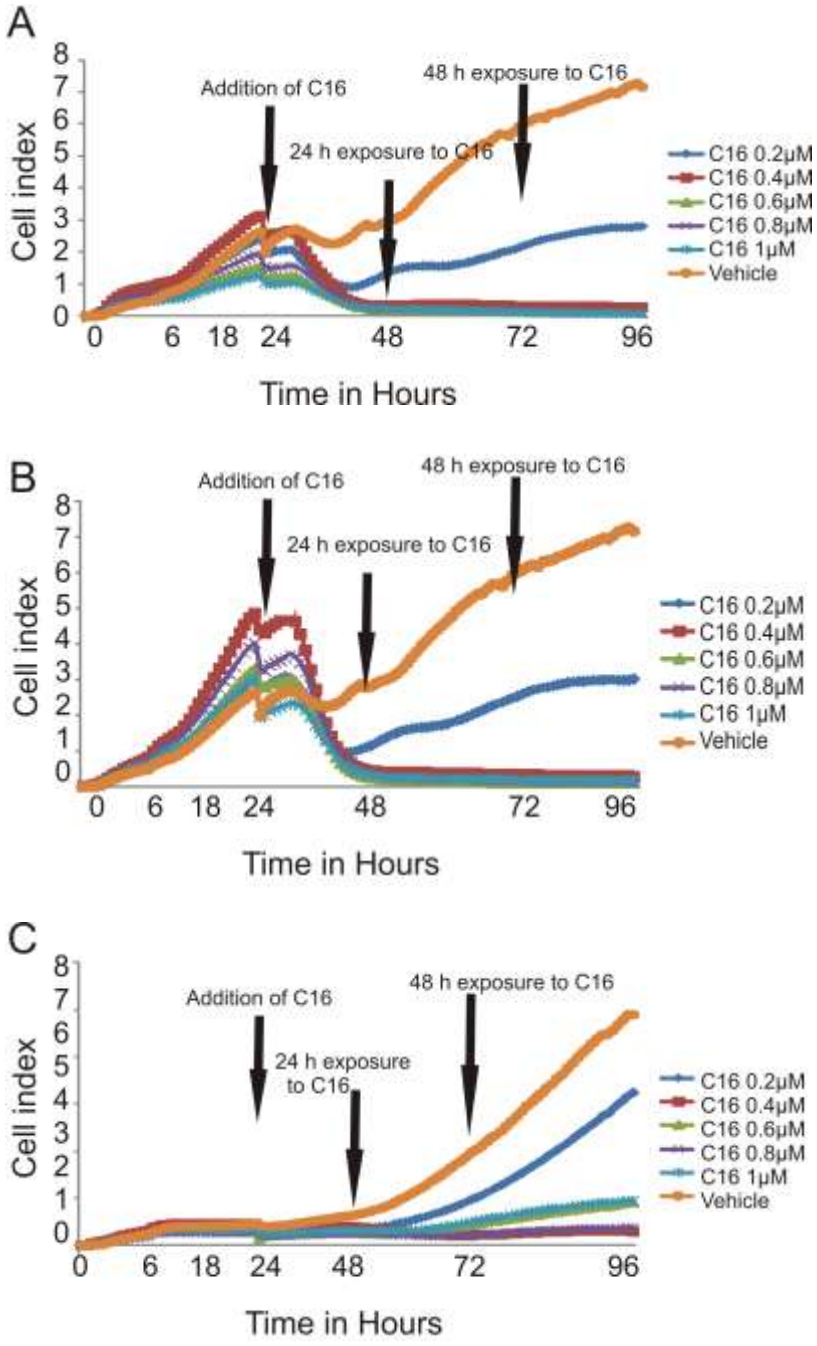


Fig. 4: The xCELLigence method demonstrated the *in vitro* effects of C16 on proliferation in MCF-7 cells (A), MDA-MB-231 cells (B) and MCF-12A cells (C). C16 had a statistically significant inhibitory effect on all treated cell lines with the MCF-7 and MDA-MDA-231 cells recovering at 0.2µM 48 h after exposure. The MCF-12A cell line recovered more effectively after 48 h when compared to the other cell lines.

## DISCUSSION

This *in vitro* pilot study investigated the effects of a novel *in silico*-designed sulphamoylated 2ME compound in an estrogen receptor negative breast adenocarcinoma cell line (MDA-MB-231), an estrogen receptor positive breast adenocarcinoma cell line (MCF-7) and an estrogen receptor negative non-tumorigenic breast cell line (MCF-12A). To date, no research regarding the *in vitro* or *in vivo* activity of C16 has been published due to the novelty of this compound. Studies performed in our laboratory revealed that other *in silico*-designed compounds inhibited the growth of various cancer cell lines in a concentration range of 0.11µM-0.22µM [16]. In the current study, C16 exerted effective antiproliferative activity at 0.2µM. In this communication, light microscopy, mitotic indices and PlasDIC demonstrated cells blocked in metaphase; several characteristics associated with apoptosis induction and decreased cell density.

It is reported in this communication that C16 exerts antiproliferative and antimitotic activity in MCF-7 cells, MDA-MB-231 cells and MCF-12A cells with the hallmarks of apoptosis present. The tumorigenic MCF-7 and MDA-MB-231 cells were more susceptible to C16 treatment compared to the non-tumorigenic MCF-12A cells. Further research will focus on the molecular signal transduction that C16 utilizes in its induction of cell death and an in-depth analysis of specific targets *in vitro* and subsequently *in vivo* investigation. The latter will contribute to the discovering of targets for cancer therapies that will aid in the design of antiproliferative- and microtubule disrupting agents.

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