**t10c12 Conjugated Linoleic Acid Suppresses HER2 Protein and Enhances Apoptosis in SKBr3 Breast Cancer Cells: Possible Role of COX2**

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Abstract

**Background:** HER2-targeted therapy with the monoclonal antibody trastuzumab (Herceptin®) has improved disease-free survival for women diagnosed with HER2-positive breast cancers; however, treatment resistance and disease progression are not uncommon. Current data suggest that resistance to treatment in HER2 cancers may be a consequence of NF-κB overexpression and increased COX2-derived prostaglandin E2 (PGE2). Conjugated linoleic acid (CLA) has been shown to have anti-tumor properties and to inhibit NF-κB activity and COX2.

**Methods:** In this study, HER2-overexpressing SKBr3 breast cancer cells were treated with t10c12 CLA. Protein expression of the HER2 receptor, nuclear NF-κB p65, and total and phosphorylated IκB were examined by western blot and immunofluorescence. PGE2 levels were determined by ELISA. Proliferation was measured by metabolism of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and apoptosis was measured by FITC-conjugated Annexin V staining and flow cytometry.

**Results/Conclusions:** We observed a significant decrease in HER2 protein expression on western blot following treatment with 40 and 80 μM t10c12 CLA (p<0.01 and 0.001, respectively) and loss of HER2 protein in cells using immunofluorescence that was most pronounced at 80 μM. Protein levels of nuclear NF-κB p65 were also significantly reduced at the 80 μM dose. This was accompanied by a significant decrease in PGE2 levels (p=0.05). Pretreatment with t10c12 CLA significantly enhanced TNFα-induced apoptosis and the anti-proliferative action of trastuzumab (p=0.05 and 0.001, respectively). These data add to previous reports of an anti-tumor effect of t10c12 CLA and suggest an effect on the HER2 oncogene that may be through CLA mediated downregulation of COX2-derived PGE2.

Introduction

Overexpression of the HER2 oncogene occurs in 25–30% of human breast cancers and is associated with poor outcome [1]. HER2 overexpression often occurs with estrogen receptor (ER) negative disease, making these tumors resistant to hormonal therapies [2]. Treatment with trastuzumab (Herceptin®) has improved disease-free survival in patients with metastatic breast cancer, but is limited by both cardiac toxicity and inherent and acquired resistance [3]. Significant effort is currently directed at combining Herceptin® with traditional anticancer agents as well as emerging therapies against additional target molecules, including inhibitors of other receptor tyrosine kinases, nuclear factor-κB (NF-κB), and chaperone protein HSP90 to improve clinical outcome [2,4,5,6].

One rationale for the use of combination therapies is to modulate multiple, deregulated tumor targets to reduce the likelihood of acquired resistance to the primary therapy. The molecular basis for acquired resistance to Herceptin® is poorly understood, but may involve HER2-independent upregulation of phosphoinositide 3 (PI3) and mitogen activated protein (MAP) kinase pathways, possibly through upregulation of insulin-like growth factor-1 receptor (IGF-1R) or EGFR ligand activation [7]. Chemotherapy-induced NF-κB expression attenuates the intended cell killing effect and may play a role in drug resistance that is often seen in HER2 and EGFR overexpression [8,9].

NF-κB is a key transcription factor in the regulation of the inflammatory response [10]. In basal conditions, NF-κB is sequestered in the cytoplasm by the inhibitor-κB (IκB) complex [11]. Activation occurs when the inhibitor of IκB, IκB-kinase (IKK) phosphorylates IκB, releasing NF-κB to migrate to the nucleus and regulate the expression of genes involved in tumor promotion and progression such as growth factors, cell cycle regulators, anti-apoptotic proteins, stromal remodeling proteases, angiogenic factors, and cell adhesion molecules [9,11,12,13]. Constitutive activity of NF-κB has been reported in a number of cancers [14,15,16,17,18,19,20] and is known to inhibit apoptosis and promote tumorigenesis through regulation of proliferation.
CLA Suppresses HER2 Protein

Materials and Methods

Reagents

Trypsin-EDTA, RPMI, PBS, fatty acid-free BSA, and DMSO were obtained from CellGrow (Hermndon, VA). Fetal bovine serum was obtained from Atlas Biologicals (Fort Collins, CO). Penicillin/streptomycin was obtained from Gibco/Invitrogen (Carlsbad, CA). Anti-beta actin, Annexin V-FTTC Apoptosis Detection Kit (APOAF), C75, HEPES buffer, DTT, MgCl₂, NaCl, and KCl were obtained from Sigma (St. Louis, MO). TritonX-100 was obtained from Atlas Biologicals (Fort Collins, CO). Penicillin/Streptomyces (Hyclone, Logan, UT), 10% FBS and 1% penicillin/streptomycin, and maintained at 57°C and 5% CO₂. Cells utilized in these experiments were confirmed to be mycoplasma free. Unless otherwise indicated, all treatments were performed in growth media.

CLA Suppresses HER2 Protein

Cell Culture

The HER2-overexpressing SKBr3 were obtained from American Type Culture Collection (Manassas, VA) and independently authenticated. Cells were cultivated in McCoy’s 5A media supplemented with 1.5 mM L-glutamine (Hyclone, Logan, UT), 10% FBS and 1% penicillin/streptomycin, and maintained at 57°C and 5% CO₂. Cells utilized in these experiments were confirmed to be mycoplasma free. Unless otherwise indicated, all treatments were performed in growth media.

Cell Viability/Proliferation/Apoptosis

Cell viability was determined by trypan blue exclusion using Beckman Coulter Counter. Proliferation was determined by metabolic activity and the reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole). For proliferation assays, 2.5×10⁵ cells/well/100 μL media were seeded in 96-well plates, allowed to adhere overnight, then washed and treated with 1×10⁻⁶ CLA or vehicle as specified in figure legends. Determination of metabolic reduction of MTT was performed according to manufacturer’s protocol (Roche Diagnostics, Mannheim, Germany). Absorbance was measured at 580 and 690 nm, using a microplate reader (Biotek Synergy 2) in accordance with the manufacturer’s protocol. Apoptosis was determined by FITC conjugated Annexin V staining according to manufacturer’s protocol (Sigma APOAF). Absorbance was read by flow cytometry using FACSan (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s protocol.

Preparation of Whole-Cell Lysates

Cell pellets were washed twice in cold PBS and centrifuged at 2000 rpm for 10 minutes, then resuspended in ice-cold RIPA buffer (1× PBS, 1% NP-40 (nonidet P-40, Sigma) 0.1% SDS) containing 1 mM phosphate inhibitor, sodium orthovanadate, and HALT protease inhibitor cocktail (Pierce/Thermo Scientific, Rockford, IL). Protein concentration was determined by Pierce Micro BCA.

Preparation of Nuclear Extracts

Cells were washed and harvested by trypsinisation, centrifuged to remove media, and washed twice in cold PBS. The protocol for nuclear extraction has been previously described [40]. All steps were performed at 4°C. Briefly, cell pellets were resuspended in hypotonic lysis buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM fresh DTT, 1× HALT protease inhibitor cocktail, and 0.1% TritonX-100) and transferred to microcentrifuge tubes. Tubes were vortexed at 16,000 rpm for 15 seconds and allowed to incubate one hour at 4°C on a rocking platform. Tubes were centrifuged at 16,000 rpm for 15 minutes, and the supernatant containing the cytoplasmic extract was removed. Nuclear pellets were resuspended in 10 μL/∼8–10×10⁶ cells nuclear extract buffer (20 mM HEPES pH7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM fresh DTT, and 1× HALT.) Samples were vortexed at the highest setting for 15 seconds and incubated on ice for 30 minutes, vortexing every 10 minutes. Tubes were centrifuges as before. Supernatant containing nuclear extracts were diluted 1:4 in storage buffer (20 mM HEPES pH7.9, 20% glycerol, 1.5 mM MgCl₂, 100 mM KCl, 0.2 mM EDTA, 0.5 mM fresh DTT, and 1× HALT.) Protein concentration was determined by Bio-Rad Assay (500-0006). Extracts were stored at −80°C until use.

Immunoblot

10–25 μg protein was loaded into 8 or 10% gels and separated by SDS PAGE using the Bio-Rad Criterion Gel system (Hercules,
Prostaglandin E2 (PGE2) Determination

Cells were grown on 12-mm glass coverslips in six-well plates at a seeding density of 4×10⁵ cells/well/3 ml. After overnight adherence, cells were treated with 40 or 80 μM t10c12 CLA or BSA for 24 hours. Cells were washed briefly in 1× PBS, 0.1% NaN₃, before fixation in 2% paraformaldehyde (Pierce) for 20 minutes, and were then permeabilized in 0.2% Tween 20 (Sigma) for 5 minutes. Coverslips were blocked in 10% goat serum for one hour at room temperature and incubated overnight with antibodies prepared in blocking buffer (1× PBS, 0.1% NaN₃, 1% Triton X). After incubation of primary antibodies, coverslips were washed 3×5 minutes in washing buffer (1× PBS, 0.1% NaN₃, 1% Tween 20). Dilutions of Alexa Fluor 488 anti-goat serum, 1% TritonX). After incubation of primary antibodies, coverslips were washed 3×5 minutes in washing buffer (1× PBS, 0.1% NaN₃, 1% Tween 20). Dilutions of Alexa Fluor 488 anti-goat serum, 1% Triton X. After incubation of primary antibodies, coverslips were washed 3×5 minutes in washing buffer (1× PBS, 0.1% NaN₃, 1% Tween 20). Dilutions of Alexa Fluor 488 anti-goat serum, 1% Triton X. After incubation of primary antibodies, coverslips were washed 3×5 minutes in washing buffer (1× PBS, 0.1% NaN₃, 1% Tween 20).

Western blot of p65 protein expression following 24-hour treatment with t10c12 CLA. Nuclear p65 was reduced at both the 40 and 80 μM doses in replicate experiments. The effect was statistically significant at 80 μM (p=0.05) when compared to untreated controls. Immunofluorescence of anti-p65 in similarly treated cells confirmed an overall decrease in the p65 protein with CLA treatment (see Figure 2B).

Under basal conditions, NF-κB is sequestered in the cytoplasm by the 1κB proteins. Phosphorylation of 1κB by IKK targets the 1κB complex for ubiquitination and proteosomal degradation, freeing NF-κB for nuclear localization [43]. To lend support to the data above, total and phosphorylated 1κB-α protein levels were measured in whole cell lysates following similar treatments. In agreement with a downregulation of NF-κB, we observed a decrease in phosphorylated 1κB protein levels at both the 40 and 80 μM dose, that was statistically significant. Figure 2C.

Treatment with t10c12 CLA or Celecoxib® Results in Decrease in PGE2 Levels and Suppression of HER2 Protein

Cyclooxygenases (COX) 1 and 2 are the rate-limiting enzymes in the conversion of arachadonic to prostaglandins. Constitutive COX1 is ubiquitously expressed and active in normal cellular processes. COX2 is induced as a consequence of NF-κB activation in response to various stimuli including stress, growth factors, cytokines, and oncogenes [13,28]. COX2-derived PGE2 has been implicated in a number of pathways involved in tumorigenesis [29] and it has been shown to be a target of CLA in a number of cell types [44,45,46]. We next asked if the apparent loss of NF-κB activity supported by the Western blots would correlate to a downregulation of COX2 activity. Supernatant obtained from the above experiments was used to measure levels of secreted PGE2 by ELISA as described in the Materials and Methods. As indicated in Figure 3A, t10c12 CLA significantly inhibited PGE2 at the 80 μM dose (p<0.05). As positive control for COX2 downregulation, cells were treated with the COX2-specific inhibitor Celecoxib® at 20 and 40 μM for 48 hours. 40 μM Celecoxib® significantly inhibited PGE2 production by more than 80% (p<0.001). Consistent with a prior report that PGE2 influences HER2 expression [29], we found that reduction of HER2 correlated to a reduction in PGE2 synthesis (Figure 3B).

The t10c12 CLA Enhances Anti-Growth Effects of TNF-α and Herceptin® in SKBr3 Cells

Overexpression of HER2 has been associated with attenuation of TNF-α-induced apoptosis through upregulation of NF-κB [47,48]. NF-κB inhibition has been shown to sensitize SKBr3 cells to TNFα [48,49]. Based on the observed inhibition of NF-κB
by the t10c12 CLA, we predicted that pretreatment with the isomer would sensitize SKBr3 to TNFα. SKBr3 cells were pretreated with 80 μM t10c12 CLA or vehicle control for 12 or 24 hours, media was removed, and cells were exposed to either TNFα in serum-depleted media (2% FBS) or serum-depleted media alone for an additional six hours. Both adherent and non-adherent cells were harvested and stained with FITC-Annexin V following the manufacturer’s protocol (Sigma APOAF), and flow cytometry was performed to measure Annexin V positive cell fraction. As indicated in Figure 4A, exposure to 80 μM t10c12 CLA showed a dose-dependent effect on the relative amount of Annexin V positive fraction following TNFα treatment that was statistically significant in 24-hour pretreatment conditions.

The monoclonal antibody trastuzumab (Herceptin®), which targets the extracellular domain of HER2, has been shown to inhibit proliferation in HER2-expressing cells, and inhibition of NF-κB has been shown to enhance these anti-proliferative effects in breast and colon cancer cells [29,50]. Though SKBr3 cells have been demonstrated to be sensitive to Herceptin®, anti-proliferative response is not generally seen before three days of exposure and is enhanced in combination with other agents [51,52,53]. Based on its inhibition of NF-κB, we hypothesized that t10c12 CLA would enhance the effect of Herceptin® in SKBr3 cells. In Figure 4B, cells were pretreated with 80 μM t10c12 CLA for 24 hours, then co-treated with 10 nM Herceptin® for an additional 24 hours. The rationale for the pretreatment was based on the observed effect of 80 μM t10c12 CLA on NF-κB downregulation in the SKBr3 cells. In preliminary dose escalation experiments, we did not detect a measureable effect of Herceptin® on proliferation in SKBr3 cells at any dose or exposure times tested (1–10 nM, 12–72 hours, data not shown). However, pretreatment with the t10c12 CLA isomer significantly enhanced the anti-proliferative effects of Herceptin® by 25% (p = 0.001) compared to vehicle (Figure 4B). Neither Herceptin® nor CLA alone effectively inhibited proliferation at the doses and time points measured.

Discussion

SKBr3 cancer cells are highly resistant to apoptosis [49,54], a phenotype that is likely due to the overexpression of HER2 [52]

Figure 1. t10c12 CLA reduces HER2 protein in SK-Br3 cells. (A) Representative western blot of total HER2 protein in response to 24 hr treatment with t10c12 CLA. Cells were plated in 6-well plates, 3 wells per treatment. Cells from 3 wells were pulled and total protein was extracted as described in Materials and Methods. 25 μg of whole cell lysate were loaded into 8% gels. Densitometry of bands was performed using Scion Image software Alpha 4.0.3.2. Expression of HER2 was normalized to beta actin and compared to vehicle treatment. Values represent the mean ±/− std error relative to vehicle from 5 independent experiments. (B) Immunofluorescence of HER2 protein in SK-Br3 cells following 24 hr treatment with 40 μM (middle panel), 80 μM (right panel) or vehicle (left panel). Cells were treated and immunofluorescence was performed as described in Materials and Methods. Images were obtained using Delta Vision deconvolution microscope with SoftwoRX 3.5.0 software at 40× and optimized using Adobe PhotoShop CS2 version 9.0.2.

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Figure 2. t10c12 CLA reduces nuclear p65 in SKBr3 cells. (A) Western blots of NF-κB p65 in nuclear extracts. Cells were plated in 6-well plates, 3 wells per treatment. Cells from 3 wells were pulled and nuclear extract was obtained as described in Materials and Methods. 10 μg of nuclear extract was loaded into 10% gels. Gel electrophoresis and immunoblots were performed as described in Materials and Methods. Densitometry of bands was performed using Scion Image software Alpha 4.0.3.2. Protein expression of p65 was normalized to total protein and compared to levels in untreated cells (vehicle only) (* = 0.05). Values represent mean +/− std error relative to vehicle control from 3 independent experiments. (B) Immunofluorescence of p65. Cells were treated as above. Immunofluorescence was performed as described in Materials and Methods. Top panel: vehicle; Middle pane: 40 μM CLA; Bottom pane: 80 μM CLA. Images were obtained using Delta Vision deconvolution microscope with SoftwoRx 3.5.0 software at 40× and optimized using Adobe Photoshop CS2 version 9.0.2. (C) Western blot of total and phosphorylated IkBα protein. Cells were plated and treated as above. 25 μg of whole cell lysate were loaded into 10% gels. Expression levels of IkB proteins were normalized to beta actin and compared to levels in untreated cells (vehicle only). Values represent mean +/− std dev from 3 independent experiments (* = 0.05). Gel electrophoresis and immunoblots were performed as described in Materials and Methods. Densitometry of bands was performed using Scion Image software Alpha 4.0.3.2.

Figure 3. Suppression of HER2 and COX2 in SKBr3 cells. Cells were treated with t10c12 CLA for 24 hrs or Celecoxib for 48 hrs. Cells were plated in 6-well plates, 3 wells per treatment. 30 minutes before collection, 15 μM arachidonic acid was added as substrate for COX2. Negative controls did not receive arachadonic acid. Cells from 3 wells were pulled for isolation of total protein and PGE2 determination by ELISA. (A) Western blots of HER2 protein following treatment with t10c12 CLA or celecoxib. Gel electrophoresis and immunoblots were performed as described in Materials and Methods. Densitometry of bands was performed using Scion Image software Alpha 4.0.3.2. (B) PGE2 levels were measured by ELISA and are presented as pg/ml relative to vehicle. Values represent the means +/− std dev. from 2 independent experiments. (* = 0.05, ** = 0.001). Experiments were performed as described in Materials and Methods.
and constitutive activity of the PI-3 kinase/AKT network, and NF-
κB-induced signaling [6,22,42]. In this study, we show evidence
that the t10c12 isomer of CLA, at physiologically obtainable doses,
significantly reduced HER2 protein in SKBr3 cells. Consistent
with previous reports of NF-κB inhibition by CLA [36,55,56],
80 μM t10c12 CLA significantly reduced NF-κB nuclear localization
and the phosphorylation of IκBα. These data are noteworthy
as they demonstrate the ability of the t10c12 CLA isomer to target
two key regulators in breast tumor promotion and treatment
resistance, the HER2 receptor and NF-κB. These results support a
mechanism of CLA that has not previously been demonstrated in
an HER2-overexpressing breast cancer cell line. Although
additional experiments are needed to confirm the direct target of
CLA’s action, we postulate two possible models for the observed
effect of CLA on HER2 protein in SKBr3 cells illustrated in
Figure 5.

In Figure 5A we suggest that CLA’s inhibition of IKK indirectly
downregulated HER2. As illustrated by the directional flow of the
diagram, inhibition of IKK results in the downregulation of NF-
κB and COX2-derived PGE2 levels. Based on published evidence
supporting an inhibitor role of PGE2 on HER2 expression [29], a
decrease in HER2 protein might also be observed by this
mechanism. CLA has been associated with a decrease in IKK
protein and activity in macrophages and attenuation of COX2
expression and PGE2 synthesis [36]. In addition to the protein
analysis performed here, additional experiments, such as kinase
activity assays for IKK α and β, are needed to confirm a direct
action of CLA on IKK. Additionally, though not included here, an
electrophoretic mobility shift assay (EMSA) would be informative
in confirming the downregulation of NF-κB transcriptional activity
that would be expected with a decrease in IKK activity.

Alternatively, the data presented here may be explained by
Figure 5B where we suggest that the observed downregulation of
NF-κB signaling is through a CLA effect on the membrane-bound
HER2 protein. This may be through a direct action causing
downregulation of protein expression, as has been demonstrated in
the HT29 colon cancer cell line [38], dissociation of HER2 from
its chaperone, HSP90, as has been demonstrated in a gastric
cancer model [57], or disruption of caveoli or lipid rafts, which has
been recently demonstrated in response to CLA [58]. Any of these
scenarios could inhibit IKK through downregulation of the PI-3
kinase pathway, resulting in decreased nuclear p65 and COX2-
duced PGE2 production, as is reported here.

Though not investigated here, CLA has been shown to
downregulate COX2 by inhibiting 12-O-tetradecanoylphorbol-
13-acetate (TPA)-induced AP-1 transcriptional activity [59]. As
AP-1 proteins c-fos and c-jun have been shown to interact with
nuclear p65 to enhance NF-κB promoter activity [60], an
alternative scenario exists in which the downregulation of
COX2 by t10c12 CLA was through an AP-1 mediated

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**Figure 4. t10c12 CLA enhances anti-growth effects of TNFα and Herceptin® in SK-Br3 cells.** (A) CLA enhances TNF-α induced apoptosis. Cells were pretreated with 80 μM t10c12 CLA for 12 or 24 hours before exposure to 50 ng/ml recombinant TNF-α for 6 hours. Cells were plated in 6-well plates, 1 well per treatment. Annexin V staining was evaluated independently for each well and values were pulled to obtain an average Annexin V value per treatment condition. FACS plots are from a single experiment. Quantification of effect is presented as mean fold change +/- std dev in % Annexin V positive staining cells relative to vehicle control and were derived from 2 independent experiments. (p = 0.05). (B) Cells were pretreated with 80 μM t10c12 CLA for 24 hrs, then co-treated with CLA +/- 10nM Herceptin® for an additional 24 hrs. Viability was assessed by MTT and absorbance at 580nm. Cells were plated in 96 well plates, 6 wells per treatment. Absorbance values were pulled for each treatment to obtain an average. Values represent the mean from 3 experiments +/- std. dev. The combination treatment significantly reduced absorbance compared to the vehicle control. Experiments were performed as described in Materials and Methods.

**Figure 5. Proposed mechanism of CLA action in SKBr3 cells.** (A) This scenario describes a direct effect on IKK by t10c12 CLA. A consequence of IKK inhibition is reduced phosphorylation of IκB and nuclear localization of p65. A decrease in COX2-derived PGE2 synthesis will result in loss of HER2 (as described in by Benoit et al, Oncogene, 2004; 23(8):1631). (B) This scenario describes a direct effect on HER2 protein by t10c12 CLA. In this scenario, HER2 is dissociated from the plasma membrane and targeted for ubiquitination and proteosomal degradation. Loss of HER2 signaling downregulates PI-3 kinase and IKK activity. NFκB is sequestered in the cytosol by IκB and its target genes such as COX2 are not transcribed.
mechanism. This possibility is supported by evidence in HER2 overexpressing breast epithelial cells in which overexpression of c-jun attenuated a pharmacological inhibition of AP-1 mediated COX2 activation [61].

There is a recognized need to develop non-toxic strategies to improve clinical outcome in HER2-positive breast cancers. Inhibition of NF-kB in combination with HER2-targeted therapies may enhance treatment response, as has been demonstrated with combination anti-COX2 and anti-HER2 therapies [50,62,63,64]. The data presented here support further investigation into anti-NF-kB agents, such as specific isomers of CLA, in combination with Herceptin® in the treatment of a subset of breast cancers that are resistant to endocrine-based therapies.

There is a prevailing interest in the potential of bioactive compounds for their efficacy in tumor prevention and treatment. CLA has been demonstrated to have potent anti-tumor effects in some animal models of breast carcinogenesis [65]. Though poorly studied in humans for the prevention of breast cancer, studies in body composition have determined it to be non-toxic at doses up to 6g/day [66]. However, studies reporting adverse effects of supplementation with mixed-isomers or the t10,c12 isomer alone suggest caution, and this is emphasized by recent reports of tumor promoting activity of the t10,c12 isomer in animal models [67,68]. Our data, however, add to a large body of work supporting an anti-tumor effect of the t10,c12 CLA isomer and warrant further investigation in the prevention and treatment of breast tumours overexpressing HER2/neu and NF-kB.

Author Contributions
Conceived and designed the experiments: MF. Performed the experiments: MF. Analyzed the data: MF. Contributed reagents/materials/analysis tools: PAT. Wrote the paper: MF. Edited and approved final manuscript: PAT.

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