Mitochondrial Topoisomerase I (Top1mt) Is a Novel Limiting Factor of Doxorubicin Cardiotoxicity

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Abstract

Purpose: Doxorubicin is one of the most effective chemotherapeutic agents. However, up to 30% of the patients treated with doxorubicin suffer from congestive heart failure. The mechanism of doxorubicin cardiotoxicity is likely multifactorial and most importantly, the genetic factors predisposing to doxorubicin cardiotoxicity are unknown. On the basis of the fact that mtDNA lesions and mitochondrial dysfunctions have been found in human hearts exposed to doxorubicin and that mitochondrial topoisomerase I (Top1mt) specifically controls mtDNA homeostasis, we hypothesized that Top1mt knockout (KO) mice might exhibit hypersensitivity to doxorubicin.

Experimental Design: Wild-type (WT) and KO Top1mt mice were treated once a week with 4 mg/kg doxorubicin for 8 weeks. Heart tissues were analyzed one week after the last treatment.

Results: Genetic inactivation of Top1mt in mice accentuates mtDNA copy number loss and mtDNA damage in heart tissue following doxorubicin treatment. Top1mt KO mice also fail to maintain respiratory chain protein production and mitochondrial cristae ultrastructure organization. These mitochondrial defects result in decreased O2 consumption, increased reactive oxygen species production, and enhanced heart muscle damage in animals treated with doxorubicin. Accordingly, Top1mt KO mice die within 45 days after the last doxorubicin injection, whereas the WT mice survive.

Conclusions: Our results provide evidence that Top1mt, which is conserved across vertebrates, is critical for cardiac tolerance to doxorubicin and adaptive response to doxorubicin cardiotoxicity. They also suggest the potential of Top1mt single-nucleotide polymorphisms testing to investigate patient susceptibility to doxorubicin-induced cardiotoxicity. Clin Cancer Res; 20(18); 4873–81. ©2014 AACR.

Introduction

Anthracycline antibiotics, and especially doxorubicin, are among the most widely used anticancer drugs (1). Their primary mechanism of action is by intercalation into DNA (2) and by trapping topoisomerase II-DNA cleavage complexes (Top2cc; refs. 3, 4) as they bind at the Top2-DNA interface (5, 6). Top2cc, in turn selectively kills cancer cells by blocking replication and transcription (4, 7–9).

Despite the efficacy of doxorubicin in pediatric (10) and adult cancers ranging from leukemia to lymphomas and solid tumors such as breast cancers (11), the main adverse effect of doxorubicin is cardiotoxicity, which can cause congestive heart failure in 30% of adults at high doses, and delayed heart failure after terminating treatment in children once they reach adulthood. The cardiotoxicity of doxorubicin appears separable from its therapeutic mechanism because cardiomyocytes are generally not replicative, and Top2α, the primary target of doxorubicin (7, 8), is not expressed in quiescent cells and undetectable in heart tissues (12). On the other hand, Top2α is required for cell proliferation and its gene TOP2A is often amplified with the HER-2 (ERBB2) oncogene in breast and other forms of cancers (13).

The cardiotoxicity of doxorubicin remains difficult to predict and is often not detected until years after the completion of chemotherapy (14). Also, the genetic determinants of doxorubicin cardiotoxicity remain unknown, at least in part, because doxorubicin cardiotoxicity is likely multifactorial and complex (15). Free radical generation is a classical mechanism by which doxorubicin injures the myocardium (16). The chemical structure of doxorubicin is prone to the generation of free radicals as doxorubicin reversibly oxidizes to a semiquinone, an unstable metabolite whose futile cycling within the mitochondria releases reactive oxygen species (ROS; ref. 17). Unfortunately, free...
Translational Relevance

Doxorubicin is one of the most widely used anticancer drugs. Yet, a significant number of patients treated with doxorubicin develop cardiotoxicity. The exact mechanisms of doxorubicin cardiotoxicity are likely multifactorial and complex, and identification of predicting factors for doxorubicin toxicity remains a clinical challenge. Here, we show that the mitochondrial topoisomerase 1 (Top1mt) is critical to limit doxorubicin cardiotoxicity. Top1mt knockout (KO) mice show hypersensitivity to doxorubicin with significant mitochondrial dysfunction, including mtDNA and cristae ultrastructure damage and respiratory chain proteins loss. Top1mt KO mice show heart muscle defects with increased death rate after treatment. Our study demonstrates the importance of mitochondrial DNA (mtDNA) regulation for doxorubicin cardiotoxicity. Deleterious genomic variants for Top1mt should be tested in patients hypersensitive to doxorubicin.

radical scavengers provide only limited heart tissue protection (18–20). The heart is selectively sensitive to reactive oxygen metabolites because of lowered antioxidant glutathione peroxidase, catalase, and superoxide dismutase levels compared with other tissue (21). An additional possibility stems from the fact that doxorubicin not only inhibits Top2α, but also Top2β. A recent study showed that genetically engineered mice lacking Top2β in their heart avoid myocardial injuries after doxorubicin treatment (22). A third possibility is the direct targeting of mitochondria by doxorubicin (23). Doxorubicin being a cationic compound readily enters mitochondria, binds to cardiolipin, and inhibits Top1mt activity in the regulatory region of mtDNA also increases 

Mitochondrion from hearts of mice were isolated following the protocol for rats published by Rogers and colleagues (33). However, Top1mt-deficient mice (Top1mt<sup>−/−</sup>) are viable, fertile, normal in size, and do not display obvious basal physical or behavioral abnormalities, indicating compensation by other topoisomerases and mitochondrial reprogramming. Indeed, Top1mt-deficient MEFs compensate their mitochondrial dysfunction by producing ATP through alternative metabolic pathways and increasing their antioxidant capacity (32).

On the basis of the fact that mtDNA lesions and radical-associated mitochondrial dysfunctions have been found in human hearts exposed to doxorubicin (26) and that Top1mt specifically controls mtDNA homeostasis (32, 33), we hypothesized that Top1mt KO mice might exhibit heart tissue sensitivity to doxorubicin.

Materials and Methods

Mouse handling

Top1mt<sup>+/−</sup> (WT) and Top1mt<sup>−/−</sup> (Top1mt KO) mice were generated from heterozygous (Top1mt<sup>+/−</sup>) and paired within the same litter. Each Top1mt KO mouse had at least one brother WT as control. Starting at 7 weeks of age, mice were treated once a week with 4 mg/kg intraperitoneal doxorubicin or saline solution control for 8 weeks. Heart tissues were analyzed one week after the last treatment. For survivals studies, mice were followed for up to 90 days after the last injection. Animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the NIH (Bethesda, MD).

Transmission electron microscopy

Mice were euthanized and heart tissues were immediately harvested and fixed in 4% formaldehyde, 2% glutaraldehyde, and 0.1 mol/L Cacodylate (pH 7.4) for 2 hours at room temperature. Small pieces of the fixed heart tissue were postfixed in 1% osmium tetroxide for 1 hour and stained in 0.5% uranyl acetate for another hour. The samples were then dehydrated in a graded series of 35%, 50%, 70%, and 100% ethanol and exchanged to propylene oxide. After infiltration at 1:1 propylene oxide and epoxy resin (Poly/Bed 812, Polysciences) overnight, samples were embedded in 100% epoxy resin. Polymerization of resin was performed for 3 days at 55°C. Thin sections of 70 to 90 nm were cut with an ultramicrotome (Leica EM UC6, Leica Microsystems), stained with uranyl acetate and lead citrate, lightly carbon coated, and imaged in a Hitachi 7650 or 7600 transmission electron microscope (Hitachi High Technologies America). Images were taken with 2k × 2k AMT digital camera (Advanced Microscopy Techniques). Mitochondrial morphometric measurements were performed using ImageJ. A total of 250 mitochondria for each condition were analyzed from 10 electron micrographs taken at ×3,000 magnification in six different sections. Two animals were used for each condition.

Mitochondria isolation

Mitochondria from hearts of mice were isolated following the protocol for rats published by Rogers and colleagues.
Mitochondrial membrane potential ($\Delta\psi_m$)

$\Delta\psi_m$ was determined in isolated mitochondria using JC-1 according to the manufacturer's protocol. Protein concentration was used for normalization.

Reactive oxygen species production measured by glutathione assay

ROS production was measured quantifying reduced glutathione (GSH) in heart tissue. GSH levels were assessed in 50 mg tissue lysates using the luminescence-based GSH-Glo Glutathione Assay Kit (Promega) according to the manufacturer's protocol.

Mitochondrial Complex IV activity

The cytochrome C oxidase activity quantification in isolated mitochondria was performed using the absorbance-based assay Mitochondrial Complex IV (Mouse) Activity Assay Kit (Millipore) and following the manufacturer's protocol. The complex IV is immunocaptured with the wells and its activity is determined by following the oxidation of reduced cytochrome c as an absorbance decrease at 550 nm.

Quantification of mtDNA copy number and mtDNA damage

For mtDNA quantification, total DNA was isolated from 30 mg of tissue using DNeasy Blood and tissues Kit (QIAGEN). Quantitative PCRs were performed in triplicates in 384-well reaction plates (Applied Biosystems). Each reaction (final volume 10 µL) contained 25 ng DNA, 5 µL of Power SYBR-Green PCR Master Mix (Applied Biosystems), and 0.5 µmol/L of each forward and reverse primer. COXI gene was amplified and β2-microglobulin (β2m) was used as normalizing control.

MtDNA damage was quantified by long-range PCR (35). A 10-kb fragment and a shorter region of mtDNA were amplified. PCR reactions were limited to 18 cycles, to ensure that amplification was still in the exponential phase. To compare mtDNA damage in each sample, PCR products were quantified with PicoGreen and the quantity of the short-range PCR product (Q) was normalized to amount of the long-range PCR product (P) measured by analysis. The damage index is determined by the ratio of Q/P.

Primers sequences used for mtDNA analysis are listed in supplementary table S1.

Western blotting

For detection of respiratory chain proteins, 50 mg of heart tissue were homogenized and lysed in radioimmunoprecipitation assay buffer (RIPA) supplemented with 0.4 mol/L NaCl and protease inhibitors (Roche Applied Science). After 1 hour at 4°C, lysates were centrifuged for 10 minutes at full speed and protein concentration in the supernatant was measured (Bio-Rad Protein Assay). Of note, 40 µg of protein were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). After 1 hour blocking with 5% milk in PBST (PBS Tween 20, 0.1%), membranes were incubated overnight with Anti-OxPhos Complex Kit antibody (#457999, Invitrogen). After three washes in PBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse (1:5,000 dilution) antibody (Amersham Biosciences) for 1 hour and then washed three times. Immunoblot analyses were detected using enhanced chemiluminescence detection kit (Pierce).

For detection of Top1 and Top2β, 100 mg of heart tissue were trimmed to size of 1 mm³, homogenized and lysed in RIPA buffer supplemented with protease inhibitors. After 1 hour shaking at 4°C, lysates were centrifuged at full speed for 10 minutes at 4°C. Supernatant was discarded and the pellet was lysed a second time for 1 hour in RIPA buffer supplemented with 0.4 mol/L NaCl and protease inhibitors. After centrifugation, proteins in the supernatant were quantified and 40 µg were subjected to SDS-PAGE as described above. The primary antibodies used were: anti-Top1 (#356597, BD Phar为准), anti-Top2β (sc-25330, Santa Cruz Biotechnology), and anti-α-tubulin (#05–829, Millipore).

Histologic analyses and immunofluorescence

Heart tissues were fixed in 10% phosphate buffered formalin, pH 7.4, at room temperature for 2 hours. Five microns sections from the paraffin-embedded hearts were stained with hematoxylin and eosin (H&E) for the analysis of nucleus hypertrophy. For cardiomyocyte cross-dimensions analysis, heart sections were deparaffinized (3 times 20 minutes in Xylene at room temperature) and fixed with 4% formaldehyde in PBS for 1 hour. After PBS washes, sections were fixed and permeabilized with prechilled (−20°C) 70% ethanol for 20 minutes and stained for 1 hour with Wheat germ agglutinin coupled to Alexa Fluor 488 (1:200; Wheat Germ Agglutinin, Alexa Fluor 488 Conjugate, Invitrogen). Tissues were then washed with PBS, and mounted using Vectashield mounting medium with 4',6-diamidino-2-phenylindole to counterstain the DNA (Vector Laboratories).

Slides after H&E staining were examined using high-resolution TV camera attached to a light microscope and the magnification was calibrated with a stage micrometer (Zeiss). Slides stained with wheat germ agglutinin were examined using a laser scanning confocal microscope (Zeiss LSM510). Images were collected and processed using the Zeiss AIM software. Nucleus size and cardiomyocyte areas were realized with ImageJ software. For each animal ($n = 4$ for each condition), four to seven regions from sections of...
the right ventricular were counted and a mean value was obtained.

Results

Lack of Top1mt increases doxorubicin-induced cardiac mitochondrial defects

To investigate the role of Top1mt in the adaptive response to doxorubicin-induced cardiomyopathy, we treated Top1mt KO (Top1mt<sup>-/-</sup>) and wild-type (WT; Top1mt<sup>+/+</sup>) mice born in similar litters from heterozygous (Top1mt<sup>+/+</sup>) parents with doxorubicin. Figure 1A shows our treatment scheme. Seven-week-old mice were treated once a week with doxorubicin at 4 mg/kg or with saline solution (control) given by injections for 8 consecutive weeks. One week after the last injection, hearts were analyzed. Additional mice were followed for survival for up to 90 days after the last injection (see below and Fig. 4).

Electron microscopy analysis of heart tissues showed no obvious difference in mitochondrial ultrastructure between WT and Top1mt KO mice treated with saline solution (Fig. 1B, left). Accordingly, surface area analysis (Supplementary Fig. S1A) and mitochondria quantitation (Supplementary Fig. S1B) showed no significant difference between WT and Top1mt KO mice, and dense and regular cristae organizations were observed in both tissues. After doxorubicin treatment, electron microscopy analyses showed Top1mt KO mice displaying more extensive mitochondrial damage compared with WT mice. Mitochondria were swollen (Fig. 1B and Supplementary Fig. S1A), and showed highly fragmented and degraded cristae (Fig. 1B). In addition, compared with WT mice, the Top1mt KO mice showed an attenuated upregulation of mitochondria number in response to doxorubicin (Supplementary Fig. S1B), indicating defective mitochondrion homeostasis in response to doxorubicin for the Top1mt KO mice.

Top1mt is required to maintain heart mitochondrial biochemical functions and mtDNA integrity after doxorubicin treatment

To determine whether the ultrastructural defects observed by electron microscopy were accompanied by mitochondrial dysfunction, mitochondria isolated from heart tissue were examined biochemically. Immunoblotting showed that doxorubicin markedly decreased the steady-state levels of complexes I, III, and IV of the respiratory chain proteins in Top1mt KO mice (Fig. 2A, right panel showing a representative heart muscle example, and Supplementary Fig. S2 for quantitaiton). Although, it is well known that complexes I and III, and especially complex IV, are depleted in heart tissue after doxorubicin treatment (36, 37), the decrease in those complexes, which are both nuclear and mitochondrial encoded, was more dramatic in Top1mt KO compared with WT mice (Fig. 2A and Supplementary Fig. S2B). On the other hand, proteins of complexes II and V, which are assembled even in the complete absence of mitochondrial protein synthesis, were unaffected (Fig. 2A). The effect of doxorubicin was specific for the heart muscle as the same...
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Figure 2. Heart mitochondria and mtDNA alterations in Top1mt KO mice after doxorubicin (DOX) treatment. A, representative Western blot analyses of respiratory chain subunits in WT and Top1mt KO mice from same litters (left, control saline injections; right, after doxorubicin). Western blot analysis shows animals from the same litter. B, cytochrome C oxidase activity in heart tissue after saline injection or doxorubicin treatment in WT and Top1mt KO mice (n = 4 for each condition). C, oxygen consumption rates of isolated mitochondria from mouse heart tissue after saline injection or doxorubicin treatment (n = 3 for saline and n = 5 for doxorubicin). D, mitochondrial membrane potential measured by staining isolated mitochondria from mouse heart tissue after saline injection or doxorubicin treatment with JC-1 (n = 3 for saline injection and n = 5 for doxorubicin treatment; *P < 0.05; t test). E, drop in reduced GSH in mouse heart tissue lysate after saline injection or doxorubicin treatment (n = 5 for saline and n = 8 for doxorubicin; ***, P < 0.006; t test). F, mtDNA copy number quantification in heart tissue after saline injection or doxorubicin treatment. mtDNA copy number was expressed relative to WT after saline injection, set as 1. Normalized intensity values are on a binary log scale (n = 6 for saline injection and n = 9 for doxorubicin treatments; ***, P < 0.006; t test). G, Left, representative agarose gel images of mtDNA long fragment (Long-F) and mtDNA short fragment (Short-F) PCR products of heart tissue after saline injection or doxorubicin treatment. Top1mt KO and WT animal from the same litters were used. Right, ratio of long fragment to short fragment PCR products quantified by PicoGreen. Normalized intensity values are on a binary log scale (n = 5 for saline injection and n = 8 for doxorubicin treatments; ***, P < 0.006; t test).

respiratory chain proteins in skeletal muscle showed no difference after doxorubicin treatment in both, WT and Top1mt KO mice (Supplementary Fig. S2A).

Complex IV activity was analyzed further by measuring cytochrome C oxidase activity in isolated heart mitochondria. Figure 2B shows that cytochrome C oxidase activity was decreased by 80% in Top1mt KO mice, whereas it decreased only by 20% in WT mice. As the final electron acceptor in the electron transport chain is oxygen, we assessed mitochondrial respiration by measuring the rate of oxygen consumption in isolated mitochondria. Oxygen consumption was decreased by about 50% in Top1mt KO compared with WT mice treated with doxorubicin (Fig. 2C). Likewise, the membrane potential in isolated mitochondria from heart tissue decreased by 31% in Top1mt KO mice (Fig. 2D). As mitochondrial
dysfunction generates ROS (38) that are quenched by GSH (39). We measured reduced GSH in Top1mt KO mice. Figure 2E shows that reduced GSH decreased by ~80% in Top1mt KO mice, whereas this level decreased by only ~40% in WT mice following doxorubicin treatment (Fig. 2E).

Each mitochondrion contains several mtDNA copies and prior observations point to the important contribution of direct and/or indirect mtDNA damage in doxorubicin cardiotoxicity (40). Accordingly, we found that doxorubicin decreased mtDNA copy number both in WT and Top1mt KO mice (Fig. 2F). However, mtDNA depletion was significantly greater in the Top1mt KO mice (Fig. 2F). Long-range PCR was also performed to evaluate mtDNA damage (41). Figure 2G shows doxorubicin-induced mtDNA damage both in WT and Top1mt KO mice. However, mtDNA damage was significantly greater in the Top1mt KO mice. The effects of doxorubicin on the mtDNA of WT mice are consistent with previous studies (24–27). However, we show here for first time that lack of Top1mt accentuates mtDNA copy number loss and mtDNA damage.

**Lack of Top1mt accentuates cardiomyocyte damage after doxorubicin treatment**

To further examine cardiomyocytes, cardiac sections were stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin, which delineate cardiomyocyte dimensions by staining glycolipids and glycoproteins enveloping individual cells (ref. 42; Fig. 3A). Doxorubicin induced hypertrophy of individual cardiomyocytes in both WT (43) and Top1mt KO mice. However, the KO cardiomyocytes were significantly larger after doxorubicin than those from WT mice (Fig. 3A and B). In addition, H&E staining showed an increased cardiomyocyte nuclear size in Top1mt KO mice (Fig. 3C and D). These results

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**Figure 3.** Doxorubicin (DOX)-induced cardiomyocyte hypertrophy and fiber damage in Top1mt KO mice. A, representative images of cardiac right ventricular sections stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin. B, quantitation of mean mitochondrial area in Top1mt KO and WT hearts (*, P < 0.05; **, P < 0.01 t test). C, representative H&E staining of longitudinal sections of the right ventricular heart. Black arrows indicate hypertrophic nuclei. D, quantification of nuclear sizes obtained by measuring the longer diameter after H&E staining (***, P < 0.0001; t test). E, representative electron microscopy images of heart muscle from WT and Top1mt KO mice after doxorubicin treatment. Left, red asterisks indicate distances between individual cardiomyocytes. Right, red arrowheads indicate myofibril defects.
demonstrate that Top1mt activity prevents doxorubicin-induced cardiomyocytes hypertrophy. To address whether cardiomyocyte hypertrophy is accompanied by defects in cardiac muscle at the ultrastructural level, we analyzed heart tissue sections from Top1mt KO and WT mice by electron microscopy (Fig. 3E). Such analysis revealed prominent defects in the hearts of Top1mt KO mice, with marked structure alterations of individual myofibrils after doxorubicin treatment. At the tissue level, the distance between individual cardiomyocytes was greater in Top1mt KO than in WT mice (Fig. 3E, left, asterisks). At the intracellular level, several prominent defects in the myofibril structure could be observed. Top1mt KO mice exhibited a range of myofibril defects, including disintegrating sarcomeres with unevenly spaced filaments “fraying” out of the myofibrils (Fig. 3E, right, arrowheads).

**Top1mt inactivation increases the lethality of doxorubicin**

In light of the accentuated heart abnormalities in the Top1mt KO mice, we followed the survival of seven pairs of animals (Top1mt KO vs. WT) for 90 days following the last doxorubicin injection. None of the animals receiving saline died, whereas doxorubicin reduced the survival of both Top1mt KO and WT mice (Fig. 4). Notably, the Top1mt KO mice showed a markedly worse survival. All 7 Top1mt KO mice (100%) died within 45 days, which is in contrast with the WT mice group where only 1 of the 7 died at day 45, and 4 WT mice remained alive at day 90.

**Figure 4.** Decreased survival of Top1mt-deficient mice after doxorubicin (DOX) treatment. Survival of mice receiving doxorubicin was assessed for 90 days after last treatment. Data are plotted as Kaplan–Meier cumulative survival curves. $P$ value was determined using the log-rank test. None of the control animals receiving saline died ($n = 7$ for each condition).

**Figure 5.** Schematic representation of the mechanism by which Top1mt influences doxorubicin (DOX) cardiotoxicity. Poisoning of Top2β by doxorubicin damages mtDNA, whereas Top1mt limits mtDNA damage.

**Discussion**

Cumulative evidence indicates the importance of mitochondrial dysfunction as a predisposing and potentially causal factor for the cardiotoxicity of doxorubicin. Our study adds novel evidence for this concept, which was recently proposed for Parkin in a myocardial infarction model (44). The difference is that Parkin is involved in mitochondrial recycling by mitophagy, whereas Top1mt is involved in mtDNA homeostasis (32).

The mechanism of mitochondrial toxicity of doxorubicin remains to be fully established. A recent study showing the involvement of nuclear Top2β (22) questioned the prior notion that doxorubicin poisons mitochondria by generating ROS. Moreover, Top2β has been shown to be present in bovine heart mitochondria (30). However, we found no evidence of Top2β overexpression to account for the hypersensitivity of the Top1mt KO mice (Supplementary Fig. S3A and S3B). Figure 5 outlines our model explaining how Top2β and Top1mt exert opposite effect on doxorubicin-induced cardiotoxicity. Although doxorubicin traps Top2β cleavage complexes, resulting in mitochondrial DNA damage and dysfunction (45, 46), Top1mt protects mitochondria (32) by maintaining normal mtDNA homeostasis and enabling damaged mtDNA to be replaced. Accordingly, tissue-specific mtDNA lesions, mtDNA copy loss, and abnormal arrangements of cristae have been found in human heart patients exposed to doxorubicin (26, 47). Mitochondrial protection is also supported as a cardioprotective strategy by recent evidence with mitochondrially targeted redox active drugs in animal models (48–50).

Our study provides the first evidence that constitutive mtDNA alterations, exemplified by Top1mt deficiency, could help identify patients at risk of doxorubicin...
Figure 6. Distribution of potentially deleterious genomic amino acid changing variants for Top1mt. Distribution of the mutations along the Top1mt polypeptide. Red: Deleterious missense variants. Blue: Essential amino acids (aa) for Top1mt activity. Green: High frequent missense variants.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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