The Angiogenesis Inhibitor TNP-470 Effectively Inhibits Human Neuroblastoma Xenograft Growth, Especially in the Setting of Subclinical Disease

Suzanne Shusterman, Stephan A. Grupp, Rosalind Barr, David Carpentieri, Huaqing Zhao, and John M. Maris

Division of Oncology [S. S., S. A. G., R. B., J. M. M.], Department of Pathology [D. C.], and Division of Biostatistics [H. Z.], The Children’s Hospital of Philadelphia, and Department of Pediatrics, The University of Pennsylvania School of Medicine [S. A. G., D. C., J. M. M.], Philadelphia, Pennsylvania 19104

ABSTRACT

Tumor vascularity is highly correlated with disease outcome in neuroblastoma. Thus, novel therapeutics that target the vascular endothelium are candidates for incorporation into clinical trials. We therefore examined the effect of TNP-470 on human neuroblastoma growth in mouse models reflecting both clinically evident and minimal disease. Mice were inoculated s.c. or by tail vein injection with 10^7 human neuroblastoma-derived CHP-134 cells and treated with TNP-470 (100 mg/kg/dose s.c. three times a week or by continuous infusion) or saline. Treatment was given as a single agent in established xenografts, 10 days after 450 mg/kg of cyclophosphamide, or 12 h after tumor inoculation. Tumor growth rate was markedly inhibited in mice receiving TNP-470 administered alone both s.c. and by continuous infusion with a treatment to control ratio (T:C) at day 16 of 0.3 (<P<0.001) and a T:C at day 30 of 0.4 (P=0.029) for each dosing method, respectively. TNP-470 also significantly inhibited tumor growth when administered following cyclophosphamide (T:C at day 30 = 0.2, P<0.001) and inhibited disease establishment when given shortly after xenograft inoculation (T:C at day 30 = 0.1, P<0.001) or tail vein injection. TNP-470 was shown to directly inhibit angiogenesis by Matrigel assay (P=0.010) and to increase the apoptotic index in treated tumors. These data show that TNP-470 is a potent inhibitor of human neuroblastoma growth rate and tumorigenicity. We speculate that TNP-470 may be a useful adjuvant therapy for high-risk neuroblastoma patients, particularly when used in settings of minimal disease status.

INTRODUCTION

Neuroblastoma is a common pediatric tumor for which the prognosis is variable and dependent on both clinical features and tumor biology (1, 2). Although low-stage disease is often successfully treated with surgery alone, approximately 50% of patients have metastatic disease at diagnosis and require intensive multimodal therapy (2, 3). Despite this aggressive treatment, the prognosis for advanced disease is poor, with a 3-year event-free survival rate of approximately 35% and significant long-term morbidity from treatment (3–5). It is therefore important to identify novel therapeutic approaches aimed at effectively treating neuroblastoma with minimal additional toxicity.

Tumor vascularity has been shown to be associated with clinical outcome in children with neuroblastoma. The vascular index is strongly correlated with prognostic markers of aggressive disease, including the presence of metastases at diagnosis, MYCN amplification, and unfavorable Shimada histopathology, as well as with decreased survival probability (6). Thus, antiangiogenic agents are a logical addition to treatment strategies for high-risk neuroblastoma patients.

TNP-470 (TAP Pharmaceuticals, Deerfield, IL) is a synthetic fumagillin analogue that interferes with angiogenesis through specific inhibition of endothelial cell proliferation and migration (7, 8). TNP-470 has been shown to be active as a single agent in a wide variety of preclinical models, including both a murine and human neuroblastoma xenograft model, and it is currently under evaluation in Phase I and II clinical trials (9–15).

In this study, we tested the hypothesis that TNP-470 would be useful in the treatment of neuroblastoma, particularly in the setting of minimal or subclinical disease to consolidate a clinical remission. We confirmed the efficacy of TNP-470 in our human neuroblastoma xenograft system and also examined a continuous infusion dosing schedule because of the drug’s short serum half-life. We then used three preclinical models to determine whether TNP-470 has enhanced efficacy when delivered in situations designed to reflect tumor remission and/or minimal disease burden. These latter experiments were designed to mimic time points in the care of high-risk neuroblastoma patients where novel therapeutic strategies are required.

MATERIALS AND METHODS

Drugs. TNP-470 was provided by TAP Pharmaceuticals and stored in the dark at 4°C. Prior to use, TNP-470 was reconstituted in sterile saline and stored in daily aliquots at −80°C. TNP-470 was used at a dose of 100 mg/kg given s.c.
three times per week or at a dose of 20, 30, or 40 mg/kg/week given by continuous i.p. infusion using an Alzet infusion pump (Alza Co., Palo Alto, CA). Cyclophosphamide (Mead Johnson, Princeton, NJ) was reconstituted with sterile water (20 mg/ml) and stored at 4°C. The dose of cyclophosphamide was 450 mg/kg divided into three doses over 6 days, given by i.p. injection (16). Dexamethasone (1 mg/kg; American Pharmaceutical Partners Inc., Los Angeles, CA) and ondansetron (3 mg/kg; Glaxo Wellcome Inc., Research Triangle Park, NC) were given s.c. 30 min before cyclophosphamide administration for gastric protection and to prevent weight loss (17, 18). Ketamine (Fort Dodge Animal Health, Fort Dodge, IA) at a dose of 150 mg/kg and Xylazine (Bayer Co., Shawnee Mission, KS) at a dose of 8 mg/kg were used for sedation and analgesia during Alzet infusion pump placement.

Cell Lines and Culture. The CHP-134 cell line was derived from the high-risk neuroblastoma and has well-characterized primary tumor of a patient with features (MYCN amplification, chromosome 1p deletion (19), and unbalanced gain of distal 17q material) that are associated with clinically aggressive disease (1). The cells were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) containing 10% fetal bovine serum and l-glutamine, 1% penicillin and streptomycin, and 0.05% gentamicin. The cells were maintained in 75-cm² cell culture flasks (Corning, Inc., Corning, NY) in a humidified atmosphere of 95% air and 5% CO₂. Cells were passaged when they reached 100% confluence.

Animals and Xenografting. Four- to 6-week-old athymic (nu/nu; NCI, Frederick, MD) and SCID/Beige (Charles River Laboratories, Wilmington, MA) mice were used. The mice were maintained in an humidity- and temperature-controlled laminar flow room and fed ad libitum. For xenografting, 10⁷ cells were pelleted and resuspended in 0.2 ml of Matrigel (Collaborative Biomedical Products, Bedford, MA) on ice. Cell suspensions and 0.1 ml of air were injected s.c. with a 26-gauge needle into the right flank of nude mice, raising a wheal. Tumor growth was observed within 10–14 days following inoculation in 95% of the animals. Tumor measurements were made by Vernier caliper three times a week and tumor volumes were calculated using the ellipsoid formula: length × width × height = 0.52 (20). For tail vein injections, 10⁷ cells were pelleted, resuspended in 0.5 ml of saline, and administered using a 26-gauge needle into restrained SCID-Beige mice. Mice were examined daily for signs of progressive disease and weights were followed three times weekly. A complete autopsy was performed on all mice which included macroscopic inspection of all organs and microscopic evaluation of the kidney, liver, lung, and spleen, as well as any other organ with gross evidence of disease. These studies were approved by the Animal Care and Use Committee of the Children’s Hospital of Philadelphia.

Alzet Infusion Pump Placement. Using sterile technique following anesthesia, a 1-cm midline abdominal incision was made and a 14-day Alzet micro-osmotic pump (0.25 µl/h, model 1002) containing either TNP-470 or saline was placed i.p. The peritoneum and skin were then secured separately using 4.0 vicryl sutures.

TNP-470 Treatment. Five separate experiments, with a total of 8–16 mice in each, were designed to evaluate the efficacy of TNP-470. Randomization to TNP-470 or placebo was balanced according to tumor size and/or mouse weight at treatment initiation. For xenograft experiments, treatment was continued until the first tumor exceeded 3.0 cm³ when all mice were sacrificed and a representative sample was autopsied. This allowed for evaluation of apoptosis and proliferation in the tumors prior to the establishment of substantial central necrosis. For the tail vein experiment, treatment was continued until the first mouse became moribund or died from progressive disease. All animals were then sacrificed and autopsied.

Histopathology. Tumors were dissected and cryopreserved in Cryomatrix (Life Sciences International, Cheshire, United Kingdom) or fixed in 10% buffered Formalin phosphate. Apoptosis was examined by TUNEL assay using the ApoTag in situ detection kit (Intergen, Purchase, NY). Briefly, tissue cryosections were fixed in 1% paraformaldehyde and postfixed in 2:1 ethanol:acetic acid. The fragmented DNA 3’OH ends were labeled with digoxigenin-dUTP, fluorescein-conjugated antidigoxigenin antibody was added, and counterstaining was done using 4’,6-diamidino-2-phenylindole. The slides were viewed by fluorescence microscopy (21). Proliferation was assessed using Ki-67 immunodetection (MIB-1; Immunotech, Marseille, France) (22). Tumor vascularity was examined using CD-31 (MEC 13.3; BD PharMingen, San Diego, CA) immunohistochemistry (23). Both stains were performed on paraffin-embedded tissue cut at 4- to 5-µm on charged slides blocked for endogenous peroxide. The Ki-67 antigen was retrieved with citrate buffer (Biogenex, San Roman, CA) in a microwave oven for 10 min. The Ki-67 antigen was visualized with an avidin-biotin method using a mouse on mouse secondary antibody (Vector Laboratories, Burlingame, CA) and diaminobenzidine as the chromogen. The CD31 antigen was retrieved with proteinase K (Dako, Carpinteria, CA) at room temperature. After blocking with normal rabbit serum, an avidin-biotin method was used with a secondary mouse-adsorbed rabbit antirat IgG antibody (BD PharMingen) and diaminobenzidine as the chromogen.

Matrigel Assay. Angiogenesis inhibition was quantified using a modification of the Matrigel assay previously described (24). Mice were injected s.c. in the abdominal midline with 0.5 ml of Matrigel alone or with 0.5 ml of 10⁷ CHP-134 cells resuspended in Matrigel. Animals were then randomly assigned to 100-mg/kg/dose TNP-470 or an equal volume of saline, three times weekly beginning 12 h after Matrigel injections. Matrigel plugs were harvested on day 13, dissolved in Matrisperse (Collaborative Biomedical Products, Bedford, MA) at 4°C, and assayed for hemoglobin content using a colorimetric assay (Drabkin method; Sigma, St. Louis, MO) (25).

Statistical Analysis. Medians and ranges of tumor volume were calculated and summarized (Table 1). Means and SE

S. Shusterman, unpublished observations.

The abbreviation used is: TUNEL, TdT-mediated dUTP nick end labeling.
for tumor volume and hemoglobin content are depicted in the figures for comparison and to show trends. Wilcoxon-Mann-Whitney exact tests were used to compare tumor growth between treatment and control groups. The same tests were used to compare hemoglobin contents between groups in the Matrigel assay. StatXact 4 for Windows was used to perform the non-parametric analysis (26).

RESULTS

Effect of TNP-470 on Neuroblastoma Xenograft Growth. In the first experiment, treatment with TNP-470 or saline by intermittent s.c. injection was initiated when the mean CHP-134 tumor volume reached 0.17 cm³ (day 0). There was a significant difference in tumor growth rate between the mice receiving TNP-470 and the mice receiving saline (Table 1; Fig. 1A, P < 0.001). The only toxicity seen was local skin irritation at injection sites in the mice receiving TNP-470. There was no weight loss in either the treatment or control animals.

A second cohort of mice was given either TNP-470 or saline by continuous i.p. infusion when the mean tumor volume reached 0.17 cm³. TNP-470 was given at three doses (20, 30, and 40 mg/kg/week). Mice receiving the highest dose of TNP-470 became severely cachectic shortly after the initiation of treatment and died by treatment day 16. Mice receiving TNP-470 at 30 mg/kg/week had marked inhibition of tumor growth but also had severe weight loss (mean, 21% of body weight). Mice receiving TNP-470 at a dose of 20 mg/kg/week had a significant inhibition of tumor growth compared to saline-treated mice (Table 1; Fig. 1B, P = 0.029). These mice had mild weight loss (mean, 12% of body weight) but no other signs of acute toxicity.

Effect of TNP-470 on Subclinical Disease. Subclinical disease was modeled in three ways. First, mice with mean tumor volumes of 0.35 cm³ were treated with cyclophosphamide (day 0). On day 10 after cyclophosphamide treatment when tumors were typically difficult to palpate, the mice were randomly

### Table 1 Effect of TNP-470 on neuroblastoma xenograft growth

<table>
<thead>
<tr>
<th>Delivery schedule</th>
<th>N</th>
<th>TD</th>
<th>Treatment, tumor volume (cm³)</th>
<th>Control, tumor volume (cm³)</th>
<th>T:C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>TNP-470 s.c.♂</td>
<td>16</td>
<td>16</td>
<td>0.6</td>
<td>0.4–1.2</td>
<td>1.9</td>
</tr>
<tr>
<td>TNP-470 i.p.♂</td>
<td>8</td>
<td>30</td>
<td>1.2</td>
<td>0.9–1.4</td>
<td>2.7</td>
</tr>
<tr>
<td>TNP-470 + CPM♂</td>
<td>16</td>
<td>30</td>
<td>0.2</td>
<td>0.1–0.2</td>
<td>1.1</td>
</tr>
<tr>
<td>TNP-470 immediate♂</td>
<td>16</td>
<td>30</td>
<td>0.2</td>
<td>0.1–0.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

♂ N, number of mice per experiment.
♀ TD, treatment day; T:C, treatment to control ratio.
♂ s.c. injection, 100 mg/kg/day, three times weekly.
♀ i.p. pump, 20 mg/kg/week.
♂ TNP-470 initiated after cyclophosphamide.
♂ TNP-470 initiated 12 h after xenografting.

Fig. 1 Effect of TNP-470 on neuroblastoma xenograft growth. Mean tumor volumes of mice with established xenografts receiving either TNP-470 or saline beginning on day 0. TNP-470 was administered s.c. at a dose of 100 mg/kg three times a week (A) or by i.p. infusion at 20, 30, or 40 mg/kg/week (B).
assigned to receive either s.c. TNP-470 or saline. There was a significant inhibition of tumor growth in mice receiving TNP-470 compared to saline (Table 1; Fig. 2A, \( P < 0.001 \)). Mice treated with TNP-470 had local skin irritation and mild weight loss (mean, 7% of body weight). Second, mice were randomly distributed to receive either TNP-470 or saline 12 h following placement of CHP-134 xenografts. The mice receiving saline had rapid tumor growth, whereas those receiving TNP-470 formed small tumors with markedly reduced growth velocity (Table 1; Fig. 2B, \( P < 0.001 \)). The only toxicity seen in this experiment was local skin irritation in mice receiving TNP-470. Third, eight SCID/Beige mice were inoculated with CHP-134 by tail vein injection and 12 h later randomly distributed to receive TNP-470 or saline three times weekly. All mice were sacrificed when the first control mouse died from tumor progression. Autopsy showed evidence of disease in three of four control mice, but no evidence of disease in the four animals treated with TNP-470 (Table 2). Neuroblastoma deposits were visualized in the kidney, liver, adrenal gland, and ovaries (Fig. 3). In addition, one mouse had neuroblastoma in the lung hilum. The mice treated with TNP-470 had severe skin irritation and 22% mean body weight loss compared to no detectable toxicity in the saline-treated mice.

**Effect of TNP-470 on Apoptotic, Proliferative, and Vascular Indices.** Autopsies were performed on a representative subset of mice from each xenograft experiment. As previously reported, CHP-134 xenografts were confined to s.c. tissues in all mice without evidence of metastatic spread (19). Tumors from mice treated with TNP-470 were typically smaller and paler in appearance (Fig. 4). Microscopically, xenografts consisted of immature neuroblasts with a high mitotic index regardless of treatment. There was a clear increase in the apoptotic index in the tumors treated with TNP-470 as compared to controls as measured by TUNEL assay. In contrast, proliferation and vascular densities by Ki-67 and CD-31 immunohistochemistry, respectively, were indistinguishable between TNP-470-treated and control tumors (data not shown).

**Effect of TNP-470 on Neovascularization.** A Matrigel assay was performed to quantitate the degree of angiogenesis inhibition by TNP-470. The hemoglobin content of each Matrigel implant harvested at day 13 was used to estimate tumor vascularity and was calculated as milligrams of hemoglobin per grams of Matrigel. Day 13 was chosen for harvest of the Matrigel plugs as this was predicted to be sufficient time for initiation of angiogenesis and was prior to exponential xenograft growth (see for example Fig. 2B). The mean weight of the

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**Table 2** Effect of TNP-470 on disease establishment in tail vein model

<table>
<thead>
<tr>
<th>Mouse (treatment)</th>
<th>Disease by location*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kidney</td>
</tr>
<tr>
<td>1 (saline)</td>
<td>−</td>
</tr>
<tr>
<td>2 (saline)</td>
<td>+</td>
</tr>
<tr>
<td>3 (saline)</td>
<td>−</td>
</tr>
<tr>
<td>4 (saline)</td>
<td>+</td>
</tr>
<tr>
<td>5 (TNP-470)</td>
<td>−</td>
</tr>
<tr>
<td>6 (TNP-470)</td>
<td>−</td>
</tr>
<tr>
<td>7 (TNP-470)</td>
<td>−</td>
</tr>
<tr>
<td>8 (TNP-470)</td>
<td>−</td>
</tr>
</tbody>
</table>

* +, neuroblastoma present; −, no evidence of disease.

b Disease present in the lung hilum.
Matrigel implants without cells was 0.10 g and the mean weight of the Matrigel implants containing cells was 0.12 g. The Matrigel implants without added cells were pale and without visible sign of vascularization. As would be predicted from their physical appearance, the hemoglobin content of the Matrigel alone was minimal, regardless of treatment (Fig. 5). In contrast, CHP-134-containing Matrigel plugs treated with saline showed a high hemoglobin content (mean, 12.8 mg of hemoglobin/g of Matrigel), whereas those treated with TNP-470 showed a significantly decreased hemoglobin content (mean, 3.2 mg of hemoglobin/g of Matrigel; $P = 0.010$).

**DISCUSSION**

Currently, children with high-risk neuroblastoma are initially treated with dose-intensive cycles of multidrug chemotherapy resulting in a response rate of approximately 75% (2). Responses to induction chemotherapy are typically consolidated with myeloablative doses of chemotherapy supported by stem cell rescue and followed by differentiation therapy with 13-cis-retinoic acid (2, 4). Despite this aggressive treatment strategy, disease relapse occurs frequently and both short- and long-term toxicities, including treatment-related acute myeloid leukemia, occur in a significant percentage of disease survivors (3–5). Clearly, novel therapeutic strategies are needed to improve the outcome of patients with high-risk neuroblastoma.

Angiogenesis inhibitors are attractive candidate agents for incorporation into high-risk neuroblastoma treatment algorithms for several reasons. First, neuroblastoma is a highly vascular solid tumor and the vascular index is correlated with advanced disease features and poor treatment outcome (6). Second, on a molecular level, increased expression of the angiogenic factors vascular endothelial growth factor, basic fibroblast growth factor, and platelet-derived growth factor as well as the endothelial integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are also associated with advanced disease features (27, 28). Last, antiangiogenic drugs have low potential for toxicity and resistance because they specifically target endothelial cells and would theoretically not add to treatment-related morbidity (16, 29).

TNP-470 is a specific antiangiogenic candidate for integration into neuroblastoma clinical trials as it has been shown in animal studies to inhibit neuroblastoma xenograft growth (11, 13, 15). Previous studies have also shown, however, that TNP-
does not cause neuroblastoma regression and efficacy is proportional to tumor volume at treatment initiation. This suggests that TNP-470 might be most effective clinically as an adjunct to conventional chemotherapy and/or in the setting of minimal residual disease following completion of standard therapy (11, 15).

In this study, we first confirmed that TNP-470 effectively inhibits neuroblastoma xenograft growth when administered as a single agent. We then demonstrated enhanced efficacy in three models designed to mimic frequently occurring clinical situations where the potential for relapse or treatment failure is high. First, using a model designed to reflect partial response to induction chemotherapy, we showed that TNP-470 clearly stabilized tumor regression following treatment with cyclophosphamide. Second, in a prevention model designed to mimic a state of minimal residual disease at the primary tumor site, we showed that TNP-470 very effectively reduced the rate of tumor growth. These data suggest that TNP-470 may be useful either between cycles of induction therapy or at the end of cytotoxic chemotherapy when many patients are known to have microscopic residual disease, but further cytotoxic drug dose escalation is not possible.

Although s.c. xenograft models have the advantage of allowing serial analysis of tumor growth kinetics, it must be noted that there are several shortcomings of this system. These include the nonphysiological s.c. location of the implanted tumor and lack of spontaneous hematogenous metastases (19). For this reason, we extended our experiments to a tail vein injection model where we showed complete inhibition of disseminated neuroblastoma formation with TNP-470, further supporting the use of this agent in the setting of subclinical disease. However, because all mice were sacrificed when the first control mouse died of progressive disease, we cannot rule out the possibility that TNP-470 delayed the time to disease progression. Additional studies in orthotopic and/or trans-
genic models of human neuroblastoma should be performed to further evaluate the efficacy of this compound in the settings of minimal disease burden.

The s.c. dose of TNP-470 used in this study was greater than previously reported, but was well tolerated and associated with a good clinical response. Recent Phase I data in adult patients shows that TNP-470 has a half-life of only 2–6 min, indicating that, rather than increasing the dose, a more frequent or continuous dosing schedule might improve efficacy (30). We therefore administered TNP-470 by continuous i.p. infusion at three dosing levels. Our data demonstrate that continuous infusion of TNP-470 is feasible and that there is similar inhibition of tumor growth as compared to conventional s.c. dosing. A direct comparison study between the two dosing regimens will be necessary to determine whether continuous infusion results in enhanced efficacy.

In our experiments, weight loss and local skin irritation were associated with TNP-470 administration, as has been reported previously (9, 11, 15). The most marked weight loss was seen in mice receiving continuous i.p. infusion of TNP-470 at the highest dose level, suggesting that this group of mice had the highest drug exposure. In adult Phase I studies, cerebellar dysfunction, characterized by dizziness and ataxia, has been the major dose-limiting toxicity seen (14, 30). We speculate that the weight loss we observed may be in part secondary to cerebellar dysfunction which made food acquisition more difficult. Of note, we did not observe additive toxicity in mice receiving TNP-470 following cyclophosphamide treatment, suggesting that TNP-470 could be used safely as an adjunct to conventional therapy. This will need to be confirmed in additional preclinical and clinical trials.

The mechanism by which TNP-470 inhibits neuroblastoma growth remains poorly defined. Although there was no quantitative decrease in xenograft vascular density, an antiangiogenic effect in vivo was clearly documented in a Matrigel assay. Tumor growth inhibition without a corresponding reduction in vascular density has been observed by others using TNP-470 as well as other angiogenesis inhibitors (23), and may simply indicate that any viable tumor requires a certain minimal vessel density for survival. In addition, as others have reported, we documented an increased apoptotic index in the treated tumors without a change in the proliferative index (21, 22). Our studies were not designed to determine the location of apoptotic cells in relation to the microvasculature, but the clustering of TUNEL-positive cells observed suggest a perivascular location, giving further evidence for an antiangiogenic mechanism. Lastly, TNP-470 was not directly cytotoxic to a panel of neuroblastoma cell lines in vitro over a several log range of concentrations.5 Further studies will be necessary to determine the exact mechanism for and cellular pathways involved in neuroblastoma tumor growth inhibition by TNP-470.

In summary, TNP-470 decreases the rate of neuroblastoma xenograft growth. In addition, TNP-470 is very effective in preventing xenograft growth in the setting of minimal disease burden. These data suggest that TNP-470 would be a useful addition to current neuroblastoma treatment strategies where induction chemotherapy failures, relapse following intensive therapy, and treatment-related morbidity remain significant clinical problems. Maximizing efficacy of this compound may require novel therapeutic approaches, such as clinical trials designed either for TNP-470 administration between cycles of induction chemotherapy or following myeloablative consolidation therapy.

ACKNOWLEDGMENTS

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REFERENCES


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