UCN-01 (7-Hydroxystaurosporine) Inhibits DNA Repair and Increases Cytotoxicity in Normal Lymphocytes and Chronic Lymphocytic Leukemia Lymphocytes

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Abstract
Elevated DNA repair processes represent resistance mechanisms to the treatment of malignancies with alkylating agents. Recently, the cell cycle checkpoint abrogator, UCN-01, was reported to inhibit nucleotide excision repair in cell-free systems. We hypothesized that if UCN-01 was combined with DNA-damaging agents, UCN-01 might inhibit the damage repair processes, thereby enhancing cytotoxicity in quiescent cells. Here, we investigated the effect of UCN-01 on DNA repair and viability of quiescent normal lymphocytes and chronic lymphocytic leukemia lymphocytes treated with UV or the cyclophosphamide prodrug 4-hydroperoxycyclophosphamide (4-HC). DNA damage repair kinetics were determined as DNA single strand breaks by the alkaline single cell gel electrophoresis (comet) assay and by [3H]thymidine incorporation. Pretreatment with UCN-01 inhibited DNA repair initiated by UV or 4-HC in normal lymphocytes as well as chronic lymphocytic leukemia lymphocytes in a concentration-dependent manner at clinically relevant levels (50–300 nM). This inhibition was demonstrated by the decreases in incision capability, DNA resynthesis, and in rejoining, suggesting that UCN-01 inhibits the multiple sites of the repair processes. The higher UCN-01 concentration (300 nM) maximized the inhibitory effects and enhanced the UV- or 4-HC-induced cytotoxicity, as determined by annexin V binding or Hoechst 33342 staining. This enhancement was not obtained by the lower concentrations that incompletely inhibited the repair, suggesting the close association between the inhibition of the repair and the enhancement of the cytotoxicity. Our findings suggest that UCN-01 may be a good candidate for combination strategies of cancer treatment.

Introduction
UCN-01 is a novel anticancer agent that was originally identified as a more specific inhibitor of protein kinase C than the parent compound staurosporine (1). UCN-01 has shown a variety of effects on the cell cycle and antiproliferative activity on growing cells (2, 3). However, the interaction of UCN-01 with protein kinase C is unlikely to be responsible for all of these effects on the cell (4, 5). As a single agent, UCN-01 blocks cell cycle progression from G1 to S-phase and induces G1 accumulation in several human tumor cell lines (4, 6). This accumulation is accompanied by a decrease in cyclin-dependent kinase 2 activity. UCN-01 also induces apoptosis in various tumor cell lines such as leukemia (5, 7), colon carcinoma (7), and lung carcinoma (8). In some cases, the induction of apoptosis by UCN-01 is correlated to the activity of cyclin-dependent kinase 1 and 2 (5, 7).

In combination treatments, concentrations of UCN-01 that are not cytotoxic abrogate arrest of the cell cycle in S or G2 after DNA damage by gamma radiation or anticancer drugs such as mitomycin C, cisplatin, camptothecin, 5-fluorouracil, and nucleoside analogues (9–14). Such abrogation of the G2 checkpoint is associated with the inhibition of the DNA damage checkpoint kinase, Chk1 (15), and is related to increased cytotoxicity. Thus, because of these unique dysregulatory effects on the cell cycle in growing cells, UCN-01 is now known as a cell cycle checkpoint abrogator, and its activity is being evaluated both as a single agent and in combination (2, 3, 16).

Recently, UCN-01 was reported to inhibit nucleotide excision repair in response to the DNA damage initiated by cisplatin (17). DNA damage induced by UV or cisplatin is generally repaired by nucleotide excision repair mechanisms (18, 19). These repair processes consist mainly of damage recognition, dual incision, excision of the oligomer including the damaged nucleotide, gap filing by DNA resynthesis, and rejoining by ligation (19, 20). UCN-01 reduced the incision activity by inhibiting the interaction between the recognition protein XPA and endonuclease ERCC1 (17).

In our previous studies, nucleotide excision repair was rapidly activated in quiescent normal lymphocytes or in CLL3 lymphocytes irradiated with UV or the cyclophosphamide prodrug 4-HC (21–23). Concurrent treatment with a nucleoside analogue permitted its incorporation into the repair patch, inhibiting repair and resulting in greater than additive cytotoxicity. In the present study, we hypothesized that if UCN-01 was combined with DNA-damaging agents or modalities that induce DNA excision repair processes, UCN-01...
would inhibit these repair processes, thereby enhancing cytotoxicity. Thus, for the present study, normal and leukemic lymphocytes were stimulated to undergo DNA repair in vitro by UV or 4-HC. DNA damage repair kinetics were evaluated by the alkaline single-cell gel electrophoresis (comet) assay. Both the formation of DNA single strand breaks resulting from incision and the subsequent rejoining event were inhibited by UCN-01 with an associated activation of apoptotic cell death processes.

Materials and Methods

Chemicals and Reagents. UCN-01 (NSC 638850; 7-hydroxystaurosporine) was kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Aliquots of UCN-01 (10 μm in DMSO) were stored at −20°C and diluted in water immediately before each experiment. The cyclophosphamide prodrug 4-HC was kindly provided by Drs. M. Colvin and S. Ludeman (Duke University, Durham, NC). This compound is metabolized to phosphoramide mustard, an active bifunctional alkylator (24, 25). [methyl-3H]Thymidine (123 Ci/mmol) was purchased from Amersham Pharma- cia Biotech (Piscataway, NJ). FITC-conjugated annexin V was purchased from Becton Dickinson (San Jose, CA). Hoechst No. 33342 was obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Normal Lymphocytes and CLL Lymphocytes. Normal lymphocytes from 6 healthy donors or leukemic lymphocytes from 16 patients with CLL that were not refractory to alkylating agents were used. Whole blood was drawn into heparinized tubes, layered over Fico/Lite LympH (specific gravity, 1.077; Atlanta Biologicals, Norcross, GA), and centrifuged at 430 g for 20 min. Lymphocytes were harvested from the interface, washed three times with fresh medium, and sized using a Z2 Coulter particle counter and size analyzer (Coulter Corporation, Miami, FL). Then, the lymphocytes were resuspended at 1 × 10⁶ cells/ml in RPMI 1640 supplemented with 10% heat-inactivated FCS (Life Technologies, Inc., Grand Island, NY) and 2 mM L-glutamine and incubated in a 5% CO₂ humidified atmosphere at 37°C overnight.

UV Exposure and Drug Treatments. Normal lymphocytes or CLL lymphocytes were preincubated with UCN-01 at indicated concentrations or not, followed by UV exposure or by an incubation with 4-HC. For UV exposure, lymphocytes (2 × 10⁶ cells) were irradiated with UV light (254 nm) from a UVGL-25 lamp (UVP, Inc., San Gabriel, CA; Ref. 21). Radiation intensity was measured by UVX-25 UV meter (UVP, Inc.). 4-HC was added to the cell suspension (1 × 10⁶ cells/ml) to give the final concentration of 60 μM, and the incubation was performed for 30 min at 37°C. The lymphocytes treated under all conditions were immediately washed into fresh medium and then incubated for the indicated periods.

Evaluation of DNA Damage Repair. DNA damage and repair kinetics were determined as DNA single strand breaks with the use of the alkaline single-cell gel electrophoresis (comet) assay as described previously (23). Briefly, after the treatment, lymphocytes were mixed with agarose and placed onto a microscope slide. After solidification, the slides were left in lysis solution (2.5 mM NaCl, 10 mM Tris, 100 mM EDTA, 10% DMSO, and 1% Triton X-100, pH 10) for 1 h at 4°C. The slides were then soaked in alkaline buffer (1 mM EDTA and 300 mM NaOH, pH 13) for 20 min and subjected to electrophoresis (35 V, 300 mA) for the next 20 min at 4°C. After the electrophoresis, the slides were neutralized and stained with ethidium bromide. Cells, 100 per treatment condition, were analyzed by a computer-based image analysis system (Kinetic Imaging Komet system, Version 4.0, Liverpool, United Kingdom). Cellular responses to DNA damage were quantified and expressed as the “tail moment.”

Evaluation of DNA Resynthesis. DNA resynthesis during the repair process was evaluated by assessing the incorporation of tritiated thymidine (21). Normal lymphocytes (2 × 10⁶ cells), having been treated previously and washed into fresh medium, were incubated with tritiated thymidine (specific activity was ~3 μCi/ml) for 0, 1, 2, 4, and 6 h. The lymphocytes were then collected, centrifuged, and resuspended in 500 μl of 0.4 n per chloric acid. The sample was mixed, centrifuged, and resuspended again in perchloric acid. After another mixing and centrifugation, the pellet was resuspended in 1 ml of 0.5 n KOH and incubated at 45°C overnight to dissolve the pellets. Radioactivity was counted with a liquid scintillation counter (Packard Instrument Co.).

Quantitation of Apoptotic Cell Death. To evaluate cytotoxicity, apoptotic cell death was determined at 24 h after the treatments as phosphatidylserine externalization using annexin V staining (23) or by the nuclear morphology using Hoechst No. 33342 staining (26). For annexin V staining, lymphocytes were suspended in 200 μl of binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, and 2.5 mM CaCl₂) and 10 μl of FITC-conjugated annexin V. The sample was incubated for 15 min at room temperature and centrifuged again. The pellet was resuspended with 500 μl of the same buffer and with 10 μl of 50 μg/ml propidium iodide. The sample was analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using the CellQuest program (Becton Dickinson). Cells positive for annexin V were considered apoptotic. For Hoechst staining, cells were incubated with 2 μg/ml Hoechst No. 33342 for 30 min at 37°C. Nuclei, 200 per treatment condition, were counted under UV illumination using the fluorescence microscope (LABOPHOTO-2; Nikon, Melville, NY). Apoptotic cell death was determined by the nuclear morphology with nuclear condensation and fragmentation.

Statistical Analysis. All statistical analyses were performed with Microsoft Excel (Redmond, WA). The extent of the incision response, the rate of repair, and cytotoxic effects were evaluated by the paired t tests with regard to the response attributable to UCN-01 and either 4-HC or UV and that observed after the combinations. All graphs, linear regression lines, and curves were generated with GraphPad, Prism software (GraphPad Software, Inc., San Diego, CA).

Results

Effect of UCN-01 on UV-induced DNA Repair in Normal Lymphocytes. To evaluate the effect of UCN-01 on DNA repair, we first used UV as a DNA-damaging modality be-
cause it induces a well-characterized nucleotide excision repair response (27). When normal lymphocytes were irradiated, the tail moment was greatest at 1 h after the exposure, suggesting that the maximal DNA strand breaks resulted from the incision of the nucleotide excision repair (Fig. 1A). The tail moment decreased promptly thereafter, suggesting that the damaged DNA was rapidly repaired and rejoined by ligation. The tail-moment value came to the control level at 4 h after UV exposure, suggesting the completion of the repair process. When the lymphocytes were preincubated for 3 h with the escalating concentrations of UCN-01, the tail-moment values at 1 h were smaller, and the subsequent tail moments at 4 h were greater than those generated by UV alone (Fig. 1A). The concentration-dependent decrease in the tail-moment values at 1 h suggests some inhibition in the recognition or the incision step (Fig. 1B). The tail-moment value at 1 h for UV alone was 5.2 ± 1.4 (mean ± SD), whereas the value for the combination with 300 nM UCN-01 was 3.6 ± 1.7, suggesting 30% inhibition (P = 0.01 for paired samples). The ratio of the tail moment at 1–4 h was taken as an indication of the rate of repair (Fig. 1C). This represents successful completion of processes enabling rejoining of the incised DNA. This was also reduced by the escalating levels of UCN-01 and came down to 1 at 150–300 nM, indicating complete inhibition. Intraindividual analyses indicated that the rate of repair at 300 nM UCN-01 was significantly less than that at 50 nM UCN-01 (P = 0.01, paired t test).

Effect of UCN-01 on the DNA Resynthesis Step in Normal Lymphocytes. The incorporation of tritiated thymidine into DNA was quantitated to evaluate the action of UCN-01 on the DNA resynthesis step. UV exposure initiated the incorporation of thymidine into DNA, and this incorporation appeared to reach a plateau at 4 h, suggesting the completion of the resynthesis step (Fig. 2). Pretreatment with UCN-01 significantly reduced DNA resynthesis at 6 h (P = 0.04 for paired samples). The average inhibition reached 45% at 6 h.

Enhancement of UV-induced Cytotoxicity by UCN-01 in Normal Lymphocytes. To evaluate the effect of UCN-01 on UV-induced loss of viability, the apoptotic cell death was determined by annexin V binding at 24 h after the cells had been treated with UV, or UCN-01, or both in combination. The combination showed more than additive apoptotic cell death than the sum of each treatment alone (paired analysis, P = 0.04; Fig. 3).

Effect of UCN-01 on 4-HC-induced DNA Repair in Normal Lymphocytes. Next, we used the prodrug of cyclophosphamide, 4-HC, as a DNA-damaging agent, because cyclophosphamide is widely used in the treatment of various malignancies including indolent diseases such as CLL (24). Unlike UV, 4-HC treatment of normal lymphocytes gave the highest tail-moment value at the end of the incubation period (Fig. 4A). This peak was followed by a rapid decrease after washing the cells into fresh medium. This suggests that 4-HC also initiated the DNA damage repair response in normal lymphocytes, and that the cells were capable of promptly initiating and rapidly completing these processes. When the
cells were preincubated with escalating concentrations of UCN-01, the initial tail-moment values were smaller, and the subsequent tail moments at 2 h were greater than those generated by 4-HC alone (Fig. 4B). The initial tail-moment value at 0 h decreased in a concentration-dependent manner, suggesting some inhibition in the recognition or the incision step (P = 0.05 for the paired analysis of 0-h tail-moment values between 4-HC alone and the combination with 300 nM UCN-01; Fig. 4C). The rate of repair, as indicated by the ratio of the tail-moment values at 0 and 2 h, was also reduced by the escalating concentrations of UCN-01 (Fig. 4D). Intraindividual analyses indicated that the rate of repair at 300 nM UCN-01 was significantly less than that at 50 nM UCN-01 (P = 0.04, paired t test).

Enhancement of 4-HC-induced Cytotoxicity by UCN-01 in Normal Lymphocytes. Apoptotic cell death was measured between 4 and 72 h to compare the cytotoxicity of 4-HC, UCN-01, or both in combination (Fig. 5). None of the treatments induced significant apoptotic morphology in nuclei after 4 h. Neither did UCN-01 alone cause cell death after as long as 72 h. The combination treatment showed more than additive apoptotic cell death than the sum of each treatment alone at 24 h (paired analysis, P = 0.04). Although 4-HC alone caused apoptosis on average 10% of the cells at 24 h, this increased to >20% at later times, thereby masking the opportunity to evaluate the effects of the combination. Thus, it appears that the combination treatment accelerates the onset of cell death; hereafter, the 24-h time point was used for the evaluation of viability.

Scheduling the Combination of UCN-01 with 4-HC. To evaluate scheduling of the combination treatment with UCN-01 in relation to DNA repair initiated by 4-HC, normal lymphocytes were incubated with UCN-01 either before 4-HC treatment or at various times thereafter. Tail moments were quantitated at 0, 0.5, 1, 2, and 4 h after washing the samples into fresh medium.

Effect of UCN-01 on 4-HC-induced Repair in CLL Lymphocytes. Finally, to evaluate this combination strategy for CLL, lymphocytes from 16 patients with CLL were treated with 4-HC, UCN-01, or both in combination. 4-HC also initiated the DNA damage repair response in CLL lymphocytes,
although there was heterogeneity among individuals (Fig. 7A). The repair process seemed most linear and steepest between 0 and 2 h, whereas the slope decreased after 2 h. Thus, the time points of 0 and 2 h were used for the subsequent comet analysis. There was a concentration-dependent inhibition of these responses by UCN-01 (Fig. 7B). The initial tail-moment values at 0 h were decreased by the escalating concentrations of UCN-01, suggesting some inhibition in the recognition or the incision step in CLL lymphocytes (P < 0.01 for the paired analysis of 0-h tail-moment values between 4-HC alone and the combination with 300 nM UCN-01; Fig. 7C). The rate of repair, indicated by the ratio of the tail-moment values at 0 and 2 h, was completely inhibited (a value of 1) by 300 nM UCN-01, suggesting inhibition of processes required for completion of repair (Fig. 7D).

Enhancement of 4-HC-induced Cytotoxicity by UCN-01 in CLL Lymphocytes. To evaluate the effect of UCN-01 on 4-HC-induced cytotoxicity in CLL lymphocytes, apoptotic cell death was determined at 24 h after the treatments. The combination of 4-HC with 300 nM UCN-01 produced more than additive apoptotic cell death than the sum of each treatment alone (paired analyses, P = 0.01; Fig. 8). This was not the case at 50 or 150 nM, the concentrations that incompletely inhibited the repair (P = 0.8 and 0.2, respectively), suggesting that the extent of DNA repair inhibition was closely associated with the enhancement of the cytotoxicity.

Discussion
In growing cells, UCN-01 has been shown to have a variety of effects by inhibiting kinases that are involved in the cell cycle progression (2, 3) and checkpoint responses (15). Our investigation was prompted by two recent reports. One is the finding that nucleoside analogues can be incorporated into the DNA repair patch generated by excision repair processes, thereby inhibiting repair and enhancing cytotoxicity (21–23). Another stimulus was the report describing UCN-01...
inhibition of nucleotide excision repair in response to the DNA damage initiated by cisplatin (17). This inhibitory effect of UCN-01 was not related to the cell cycle progression that has been a major target for the study of the activity UCN-01. Therefore, we have used quiescent lymphocytes to eliminate the influence of the cell cycle to focus on different actions of UCN-01 in the context of DNA repair processes.

DNA repair initiated by either UV or 4-HC was inhibited by UCN-01 in normal lymphocytes as well as CLL lymphocytes in a concentration-dependent manner. The inhibitory effects were demonstrated by the decrease in the initial formation of the tail moment, the inhibition of thymidine incorporation, and the reduction of the overall rate of repair. These parameters are thought to reflect the incision around the lesion, DNA resynthesis, and the ligation steps of excision repair processes (22, 23, 28). The cytotoxicity induced by UV or 4-HC in the presence of 300 nM UCN-01, the concentration that maximized repair inhibition, was greater than that predicted by additivity of the agents alone. Thus, these findings extend prior studies (17) that demonstrated the inhibitory action of UCN-01 on nucleotide excision repair to 4-HC, an agent that induces multiple excision repair mechanisms.

Damage to DNA caused by UV is composed of cyclobutane dimers and 6-4 photoproducts, which activate nucleotide excision repair processes (19, 20, 27, 29). In contrast, alkylation with agents such as 4-HC has been shown to induce base excision repair and cross-link repair as well (18, 30, 31). In the present study, UV induced a significant increase in a tail-moment value at 1 h after the exposure (Fig. 1), whereas the cellular response to 4-HC was maximal at the end of the 30-min incubation (Fig. 4). Because this initial elevation represents the recognition and subsequent incision steps in the repair processes, the results are consistent with the interpretation that 4-HC generates responses earlier than UV by activating multiple mechanisms of recognition and incision as detected by the comet assay. The subsequent gap filling by DNA resynthesis after irradiation was demonstrated by the incorporation of tritiated thymidine into DNA (Fig. 2). This increased time dependently and reached a plateau at 4 h, suggesting the completion of this step. This behavior might be associated with the decrease in the tail-moment values in later times. After the initial increase, the tail-moment values declined with time, decreasing to the control level at 4 h after UV exposure. This diminution of the tail-moment values represents the disappearance of the DNA single-strand breaks that had been incised in the initial steps of the repair processes. Therefore, the rate of repair, the ratio of the tail-moment values at 1 h to those at 4 h, would represent the sum of processes leading to the rejoining of the DNA strands.

UCN-01 at 300 nM maximally inhibited DNA repair initiated by both UV and 4-HC and enhanced the cytotoxicity in both normal and CLL lymphocytes (Figs. 3, 5, and 8). The inhibition of the repair by the higher concentration (300 nM) of UCN-01 was significantly greater than that caused by the lower concentration (50 nM). Lesser concentrations of UCN-01 that incompletely inhibited repair did not show greater than additive cell killing (Figs. 7 and 8). The actions of UCN-01 were also schedule dependent, because the lymphocytes treated with UCN-01 after completion of the repair processes neither formed comets nor did cells become annexin positive (Fig. 6). These findings suggest that the inhibition of repair would be closely associated with the enhancement of cytotoxicity. Moreover, annexin-positive lymphocytes at 4 h after the treatment were <10%, indicating no increased loss of viability (Fig. 5). Therefore, we speculate that the inhibition of the repair would be attributed to the specific effect of UCN-01, not to an immediate cytotoxic action of the compound, which has been observed only at considerably greater concentrations after durations of days (32, 33). Investigations of the signaling pathways that are activated by inhibition of DNA repair processes have been initiated (34). A recent Phase I trial of UCN-01 by 72-h continuous infusion indicated that a maximum tolerated dose of 42.5 mg/m²/day for 3 days achieved the free drug concentration at 111 nM, measured as a salivary concentration as a reflection of plasma levels (16). A second Phase I investigation using a 3-h i.v. infusion of 51.1 mg/m² demonstrated free UCN-01 in plasma to exceed 400 nM. This suggests that with infusion schedule modifications, the UCN-01 concentrations inhibitory to DNA repair used in the present study may be clinically achievable. Moreover, the elimination half-life of the plasma UCN-01 concentration was extremely long with the median value of 26 days (16). This indicates that an inhibitory action on DNA repair by UCN-01 could be effective for a prolonged duration, and in fact this might extend to a subsequent course of therapy.

With regard to scheduling relative to induction of DNA repair, only pretreatment with UCN-01 inhibited 4-HC-induced DNA repair and enhanced the cytotoxicity (Fig. 6). This is similar to the requirements for cytotoxicity observed when 4-HC-induced repair in CLL lymphocytes was inhibited by the nucleoside analogues fludarabine and clofarabine (23). Apparently, both normal and leukemic lymphocytes are primed with excision repair processes to respond to DNA damage. This immediate response capability necessitates, however, the presence of the repair inhibitor in cells at the time of damage to maximize the consequences of this mechanism-based interaction. Although UCN-01 alone is clearly toxic to CLL lymphocytes, the greater concentrations and longer duration of incubations required for these actions suggest a different mechanism of action (32, 33).

The precise target(s) of UCN-01 in the DNA excision repair processes remains to be elucidated. The report of Jiang and Yang (17) demonstrated that UCN-01 inhibited the interaction of XPA and ERCC1 and reduced the incision activity in assays of nucleotide excision repair. This cannot fully explain the actions of UCN-01 on DNA repair demonstrated in the present study, because clearly the processes of repair that occur subsequent to incision are also affected. Prior investigations have demonstrated that UCN-01 inhibits the phosphotransferase activity of multiple kinases (1, 15, 35, 36). Most likely, this is attributable to UCN-01 blocking access of ATP to its binding site on the enzymes. In this context, several proteins involved in nucleotide excision repair exhibit 4 Y. Sasaki, personal communication.
kinase activity and therefore might be considered as potential targets for UCN-01. For instance, replication protein A becomes hyperphosphorylated in response to DNA damage induced by UV (37–39). Gap filling and ligation is mediated via an interaction between proliferating cell nuclear antigen and replication factor C. Proliferating cell nuclear antigen interacts with cyclin-dependent kinase 2 and cyclin A, which target replication factor C and DNA ligase I for phosphorylation (40–42). Cyclin-dependent kinases 1 and 2 might be dysregulated through the inhibition of Chk1 by UCN-01 (2, 3, 43). The biological significance of the inhibition of such phosphorylation reactions is not completely understood. However, some of the DNA repair proteins must be in the proper phosphorylation states for the optimal efficiency of the excision repair (20). Thus, it is suggested that a protein kinase inhibitor such as UCN-01 might interfere with phosphorylation of repair enzymes, resulting in the inhibition of the repair, although this remains to be determined.

In conclusion, UCN-01 has shown several effects on the cell cycle, which would address the use of this drug in combination strategies for cancer treatment in terms of the cycling cells. The present study demonstrated that UCN-01 inhibited DNA repair, thereby enhancing the cytotoxicity of DNA damage-inducing agents in quiescent cells. Our findings suggest that UCN-01 would be a good candidate for combination strategies in slow-growing tumors such as CLL.

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References


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