Cell Cycle Checkpoint Abrogator UCN-01 Inhibits DNA Repair: Association with Attenuation of the Interaction of XPA and ERCC1 Nucleotide Excision Repair Proteins

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Abstract

UCN-01, an antitumor agent currently in Phase I clinical trials, has been found to potentiate the cytotoxicity of cisplatin (CDDP). Because mammalian cells remove CDDP-induced DNA adducts through the nucleotide excision repair (NER) pathway, we determined the effects of UCN-01 on NER by measuring its effects on the interaction of the repair factors XPA and ERCC1 and the phosphorylation/dephosphorylation of the repair proteins. The repair activity, as measured by an in vitro repair synthesis assay and an in vivo host-cell reactivation assay using A549 cells, was significantly reduced. Although expression of XPA and ERCC1 proteins was elevated in cells exposed to UCN-01, the treatment resulted in a decreased ERCC1 level in the Triton X-100-insoluble fraction of cell lysates. A pull-down assay using the MBP-XPA fusion protein showed a significant reduction in the binding of ERCC1 to XPA in nuclear extracts from UCN-01-treated cells compared with untreated cells, suggesting that UCN-01 reduced the XPA-ERCC1 interaction. Consistent with these data, lower repair incision activity was found in the cell extracts from UCN-01-treated cells. In vitro phosphorylation revealed that UCN-01 had no effect on the phosphorylation/dephosphorylation status of either XPA or ERCC1; however, UCN-01 caused dephosphorylation of an unidentified XPA-bound protein with an apparent molecular mass of 52 kDa. Taken together, these data demonstrate the NER-inhibitory action of UCN-01, which is associated with the inhibition of the XPA-ERCC1 interaction by UCN-01 and with the effect of UCN-01 on the phosphorylation/dephosphorylation of an XPA-bound, 52-kDa protein, the identity of which remains to be determined.

Introduction

The cell cycle checkpoint abrogator UCN-01 (1-3), an antitumor agent presently in Phase I clinical trials (4, 5), produces synergistic cytotoxicities when administered in combination with the DNA-damaging agent CDDP (6). The exact mechanisms by which UCN-01 enhances the sensitivity of cells to CDDP remain largely unclear. Because arrest of the cell cycle after DNA damage, particularly in the G2 phase, is believed to promote DNA repair before cells enter mitosis, abrogating cell cycle arrest presumably shortens the repair time and may thereby result in inefficient DNA repair. Evidence supporting this contention comes from the recent observation by Bunch and Eastman (3) that UCN-01 is capable of abrogating CDDP-induced G2 arrest in CHO cell lines, regardless of their DNA repair status; however, UCN-01-enhanced CDDP cytotoxicity is found only in repair-proficient CHO/AA8 cells, not in repair-deficient CHO/UV41 cells. Although such evidence supports a link between UCN-01 and DNA repair, it remains to be determined whether UCN-01 directly affects the repair apparatus or indirectly interferes with the repair signaling pathway.

In exploring this question, we have focused on the NER pathway, because mammalian cells repair CDDP-induced DNA intrastrand adducts via this pathway, which involves the coordinated action of more than 30 polypeptides (7). There are two distinct functional modes of NER. One is global genome repair, which occurs throughout the genome. The other is TCR, which preferentially repairs the template strand of active genes transcribed by RNA polymerase II. In global genome repair, binding of the XPA recognition protein to the DNA lesion sites is an important early step of the pathway. The interaction of XPA with the 34-kDa subunit of RPA (RPA34) at DNA lesion sites activates XPA to recruit other NER components, such as the basal transcription factor TFIIH complex and ERCC1/XPF heterodimeric excinucleases. TFIIH contains nine subunits, including XPB (p89), XPD (p80), TFB1 (p62), and TFB2 (p52). The recruitment of the TFIIH multisubunit complex activates the intrinsic helicase activities of XPB and XPD that initiate the opening of DNA and facilitate ERCC1/XPF and XPG excinucleases to make dual incisions on the phosphodiester bonds at 22 to 24 nucleotides to the 5′ side and 4 to 6 nucleotides to the 3′ side of the damage site (8). In TCR, the coupling factor CSB interacts with TFIIH and RNA polymerase II, resulting in the recruitment of TFIIH to the stalled polymerase II at the lesion site (9, 10). TFIIH then interacts with XPA and brings XPA proximal to the lesion site (11). Although the modes of damage recognition in global genome repair and TCR are different, the XPA protein is equally important to both pathways (12). Because of the central role that XPA plays in DNA lesion recognition and its interaction with other NER repair proteins, the XPA-involved protein interaction network is essential in regulating the early stages of NER. Therefore, factors that affect their interactions are likely to alter cellular repair efficiency.

Another candidate for mediating UCN-01-CDDP synergistic cytotoxicity is ERCC1. The level of expression of the ERCC1 gene has been shown to be associated with the sensitivity of tumor cells to CDDP (13). This 15-kb repair gene is located on human chromosome 19. Its gene product forms a heterodimer with XPF, and the ERCC1/XPF complex cleaves the damaged strand at the 5′ end of the lesion. Using an in vitro complementation assay, Park and Sancar (14) determined that the XPA-bound fraction of HeLa cell-free extracts complemented the excinuclease activity in the cell extracts from mutant cells that were defective in either ERCC1, XPF, and XPA repair protein and concluded that XPA forms a ternary complex with...
ERCC1 and XPF. In fact, the direct interaction of XPA and ERCC1 has been delineated using a yeast two-hybrid system and in vitro binding assay (15). Because XPA and ERCC1 are known to play essential roles in the recognition and incision of CDDP-damaged DNA and because UCN-01 is able to alter the expression and/or posttranslational modification of gene products (16), we investigated whether XPA and ERCC1 are targets of the NER-inhibitory action of UCN-01. In this study, we demonstrate by an in vitro repair synthesis assay and an in vivo host-cell reactivation assay that exposure to UCN-01 led to suppression of repair of CDDP-induced DNA lesions. The inhibition of the repair activity was associated with an attenuation of the XPA-ERCC1 interaction mediated by UCN-01.

Materials and Methods

Chemicals and Cell Culture. UCN-01, obtained from the Drug Synthesis and Chemistry Branch, NIH (Bethesda, MD), was dissolved in DMSO as a 10 mM stock solution and diluted just before each experiment. CDDP was purchased from Bristol-Myers Squibb (Princeton, NJ). Kloenow fragment and restriction enzymes were purchased from Life Technologies, Inc. (Gaithersburg, MD). Antibodies, unless otherwise specified, were from Santa Cruz Biotechnology (Santa Cruz, CA). A549 human non-small cell lung carcinoma cells were from the American Type Culture Collection (Rockville, MD) and were maintained in DMEM supplemented with 10% FCS.

Intracellular Drug Accumulation and DNA Platination. A549 cells, grown to 80% confluence, were exposed to CDDP (0.15, 0.30, or 1.0 mM) for 2 h as a single agent or in combination with UCN-01 (0.50 mM UCN-01 for 0.15 and 0.30 mM CDDP, or 250 mM UCN-01 for 1.0 mM CDDP). After the drug was washed off, the cells were incubated with growth medium containing the same concentrations of UCN-01 for an additional 22 h (for 50 mM UCN-01) or was washed off, the cells were incubated with growth medium containing the undamaged pHM control plasmid (a gift from Dr. R. D. Wood, The Rockefeller University, New York). In fact, the direct interaction of XPA and ERCC1 has been delineated using a yeast two-hybrid system and in vitro has been delineated using a yeast two-hybrid system and in vivo host-cell reactivation assay that exposure to UCN-01 led to suppression of repair of CDDP-induced DNA lesions.

The repair synthesis assay was adapted from the method of Hansson and Wood (17), as described previously (18). The repair activity was measured by the incorporation of [α-32P]dATP into a CDDP-modified closed circular plasmid using whole-cell extracts from UCN-01-treated (50–200 nM; 24 h) and untreated A549 cells. Aliquots containing 300 ng each of CDDP-damaged pB9 (pBlueScript KS++; Stratagene, La Jolla, CA) and an undamaged pHM control plasmid (a gift from Dr. R. D. Wood, Imperial Cancer Research Fund, South Mimms, United Kingdom) were incubated with 100 μg of whole-cell extract in a reaction mixture containing [α-32P]dATP for 5 h at 37°C. At the end of incubation, plasmid DNAs were purified by phenol/chloroform extraction, linearized with BamHI, and subjected to electrophoresis. The incorporation of radioactivity was quantified after normalization for gel loading. DNA repair activity was determined by subtracting the nonspecific incorporation of [α-32P]dATP measured in the undamaged pHM control from the total incorporation measured in the damaged pB9 substrate.

Host-Cell Reactivation Assay. One set of cells was transfected with a platinated pSV2-CAT plasmid (8 μg) using lipofectin (Life Technologies, Inc.) according to the manufacturer’s instructions. Transfected cells were incubated with UCN-01 for 14 h to measure its effect on the repair. Another set of cells was processed in parallel, except that undamaged pSV2-CAT was used for the transfection. CAT activity was determined under standard conditions using 30 μg of cell lysate and using [3H]chloramphenicol as the substrate. To quantify the effect of UCN-01 on repair activity, we first normalized CAT activity for the effect of UCN-01 on the enzyme expression measured in the cells transfected with the undamaged plasmid, and the repair activity in the cells transfected with platinated pSV2-CAT plasmid was then expressed as the percentage of that in the untreated control, in which CAT expression was derived from the damaged reporter gene without any influence of UCN-01.

Quantification of Repair Incision Activity. Incision activity was measured by the incorporation of Kloenow enzyme-catalyzed, radiolabeled dATP into the excised repair intermediate, which was purified from the incorporation reaction as described previously (18). Briefly, the incision reaction was carried out like the in vitro repair synthesis assay, except that the progression of DNA repair synthesis was blocked by adding aphidicolin and limiting deoxynucleotidetriphosphate in the reaction mixture. Under these conditions, an excised repair intermediate was generated from CDDP-modified pB9. After DNA purification, this repair intermediate was isolated in a reaction mixture containing 90 mM HEPES-KOH, 2 mM MgCl2, 2 mM DTT, 20 μM each of dGTP, dCTP, and dTTP, 4 μM dATP, 2 μCi [α-32P]dATP, and 1 unit of Escherichia coli DNA polymerase I, large (Klenow) fragment. At the end of incubation (20°C for 10 min), the plasmid DNA was purified, linearized by BamHI, and subjected to electrophoresis. The incision activity was quantified by measuring the specific incorporation of radioactive dAMP into the nicked pB9 after normalization for the DNA amount and subtraction of the nonspecific incorporation measured in the undamaged pHM.

Isolation of Detergent-insoluble Proteins. A549 cells, grown to subconfluence, were incubated with CDDP (1 mM) or combined CDDP (1 mM) and UCN-01 (50, 100, or 250 mM) for 2 h, followed by a second incubation with UCN-01 alone for 3 h. At the end of the second incubation, an aliquot of the collected cells was saved for preparation of the whole-cell lysates. The remaining portion was pelleted, resuspended in three to five volumes of a lysis buffer [50 mM Tris-HCl (pH 7.4), 0.25 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM DTT, 0.1 mM Na3VO4, 50 μg/ml PMSF, 15 μg/ml leupeptin, 10 mM benazmidine, 1 μg/ml aprotinin, and 1 μg/ml pepstatin] for 30 min before centrifugation at 13,000 × g for 10 min at 4°C (19, 20). The extraction was repeated once, and the pellets were reconstituted in Laemmli buffer. Aliquots of whole-cell lysates and the detergent-insoluble fraction were subjected to Western blot analysis using an anti-XPA polyclonal antibody, and the protein bands were visualized by ECL (Amersham, Arlington Heights, IL). The whole-cell lysate and detergent-insoluble fraction containing ERCC1, p62, and p89, and RPA34 were also assayed by Western immunoblotting using monoclonal anti-ERCC1 (PharMingen, San Diego, CA), anti-p62, and anti-p89 antibodies and anti-RPA34. The membranes were probed with anti-β-actin monoclonal antibody (Amersham) after the antibody was stripped off. The protein expression after normalization with β-actin is presented as the percentage of that in the CDDP-treated control, which was assigned a value of 100%.

MBP Pull-Down Assay. The plasmid construct pMAL-XPA was a gift from Dr. Aziz Sancar (The University of North Carolina, Chapel Hill, NC). To construct pMAL-ERCC1, a full-length ERCC1 cDNA was first amplified by PCR (Tag Plus Long PCR system; Stratagene) using 5′-ATTAAGCTTG-GGCGCTGGGTCATAG-3′ and 5′-TCCGAAATTCGAGGACCTGGAAG-3′ as the primers and pCD plasmid carrying ERCC1 cDNA (provided by Dr. J. H. Hoeijmakers, Erasmus University, Rotterdam, the Netherlands) as the template. The PCR-amplified fragments were blunt ended with Kloenow fragment, ligated with EcoRI, and inserted into pUC18 (Life Technologies, Inc.). The EcoRI/HindIII fragment of the pUC18 construct containing ERCC1 cDNA was then subcloned into pMAL-c2X vector (New England Biolabs, Beverly, MA). The recombinant fusion proteins were prepared by expressing pMAL-XPA or pMAL-ERCC1 plasmid in E. coli BL21 (DE3) induced by isopropyl-1-thio-β-D-galactopyranoside, and the fusion proteins were purified with amylose resin according to the manufacturer’s recommendation (New England Biolabs). For preparation of nuclear extracts for the pull-down assay, nuclei were isolated from A549 cells treated with UCN-01 for 5 h or untreated. Briefly, 105 cells were suspended in 0.4 ml buffer A [10 mM HEPES (pH 7.4), 10 mM KCl, 2 mM MgCl2, 1 mM DTT, 0.1 mM EDTA, 1 mM Na3VO4, 50 μg/ml PMSF, 15 μg/ml leupeptin, 10 mM benazmidine, 1 μg/ml aprotinin, and 1 μg/ml pepstatin] on ice for 15 min before 25 μl of 10% NP40 were added, and the incubation continued for an additional 30 min. The nuclei were pelleted, suspended in 0.4 ml binding buffer [10 mM HEPES (pH 8.0), 2% glycerol, 50 mM KCl, 50 mM MgCl2, 0.5 mM DTT, 1 mM EDTA, 1 mM Na3VO4, and the protease inhibitors as described above], and sonicated to near clarity. The supernatant fraction after centrifugation was collected as the nuclear extracts. The MBP pull-down assays were performed as described by Li et al. (15) with minor modifications. Briefly, a 10-μg aliquot of MBP fusion protein immobilized on the resin was incubated with 200 μl of nuclear extract containing target proteins for 1 h at 4°C. After three washes with binding buffer, the bound proteins were dissociated from the resin by boiling in Laemmli buffer, resolved on 10% SDS-PAGE, and then analyzed by immunoblotting.
**In Vitro Phosphorylation.** The method described by Shimizu et al. (21) was adapted for the in vitro phosphorylation experiment. A549 cells were suspended in a cell lysis buffer containing 1 mM KH₂PO₄, 150 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM Na₃VO₄, 5 mM HEPES (pH 7.4), 10% glycerol, 0.3% Triton X-100, 50 μg/ml PMSF, 1 μg/ml aprotinin, and 15 μg/ml leupeptin, sonicated, and set on ice for 15 min before centrifugation (13,000 × g for 5 min at 4°C). A 500-μg aliquot of supernatant was added to the reaction mixture (final volume, 200 μl) containing 10 μM [γ⁻³²P]ATP and 10 μg of resin-immobilized MBP-ERCC1 or MBP-XPA. After incubation at 37°C for 10 min, the resin-bound proteins were pelleted, washed with the lysis buffer four times, and resolved on SDS-PAGE. Protein phosphorylation was measured by the incorporation of radioactive phosphates and visualized by autoradiography of the gel.

**Results**

**Effect of UCN-01 on DNA Repair.** That UCN-01 affects DNA repair activity was first suggested by the flameless atomic absorption spectrophotometry results, which indicated that UCN-01 at 50 and 250 nM enhanced DNA platination in A549 cells up to 71% when compared with the untreated control (Table 1). The increase cannot be explained by an enhanced cellular uptake of CDDP because the intracellular platinum level was not affected by CDDP treatment. The effects of UCN-01 on the repair activity were further assessed by an in vitro repair synthesis assay. In the extracts from UCN-01-treated A549 cells, the repair activity was significantly reduced when compared with that in their untreated counterparts; the inhibition was UCN-01 dose dependent (IC₅₀, 125 nM; Fig. 1A).

In the in vivo host-cell reactivation assay, the repair activity was measured by the reactivation of the CAT activity in the host A549 cells transfected with a CDDP-damaged pSV2-CAT plasmid DNA. After normalization for the effect of UCN-01 on the reporter gene expression, the repair activity was significantly inhibited by UCN-01 (IC₅₀, 78 nM; Fig. 1A).

**Effects of UCN-01 on the Expression of XPA and ERCC1 and on Their CDDP-induced Subcellular Translocation.** We investigated two key repair factors involved in damage recognition and repair incision as potential targets for the effects of UCN-01 on the NER pathway. The results from Western blotting show that exposure of the CDDP-treated cells to UCN-01 induced not only an increase in XPA expression (Fig. 2, Lanes 3–5) but also an accumulation of XPA protein in the detergent-insoluble fraction of the cell lysate (Fig. 2A, Lanes 8–11, and Fig. 2C). In contrast, no significant amount of XPA was detected in the detergent-insoluble fraction of lysates from the untreated cells (Fig. 2A, Lane 7) or from the cells treated with UCN-01 alone (Fig. 2A, Lane 12). Such results suggest that the translocation of XPA into the detergent-insoluble fraction is DNA damage dependent. The proteins associated with the detergent-insoluble fraction presumably represent the proteins that are bound to DNA (10).

**Table 1** Effect of UCN-01 on intracellular platinum accumulation and DNA platination in A549 cells.

<table>
<thead>
<tr>
<th>CDDP (nm)</th>
<th>UCN-01 (nm)</th>
<th>Pt in whole cells (ng/mg protein)</th>
<th>Pt in DNA fraction (ng/mg DNA)</th>
</tr>
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<tbody>
<tr>
<td>0.15</td>
<td>0</td>
<td>110 ± 14</td>
<td>26 ± 3</td>
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<tr>
<td>0.15</td>
<td>50</td>
<td>98 ± 8</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>0.30</td>
<td>0</td>
<td>168 ± 11</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>0.30</td>
<td>50</td>
<td>172 ± 15</td>
<td>88 ± 3</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>430 ± 28</td>
<td>436 ± 20</td>
</tr>
<tr>
<td>1.0</td>
<td>250</td>
<td>440 ± 8</td>
<td>744 ± 29</td>
</tr>
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*Mean ± SD from three independent experiments.

UCN-01 also elevated ERCC1 expression (Fig. 2, Lanes 3–5). However, unlike XPA, ERCC1 in the detergent-insoluble fraction was decreased as a result of UCN-01 treatment (Fig. 2A, Lanes 8–11, and Fig. 2C). Thus, it appears that UCN-01 induces an increase in DNA-bound XPA without a simultaneous recruitment of ERCC1. The decrease in the DNA-bound ERCC1 with UCN-01 exposure was not likely due to altered nuclear compartmentation of ERCC1 because the nuclear ERCC1 level was not affected by UCN-01, as determined by Western blotting of nuclear extracts (data not shown). By eliminating this possibility, the results support the possibility of an alteration in the interaction of XPA and ERCC1.

**Effect of UCN-01 on the XPA-ERCC1 Interaction.** A pull-down assay was used to determine the effect of UCN-01 on the interaction of XPA and ERCC1 by directly measuring their binding. To determine the XPA-binding activity of ERCC1, nuclear extracts from cells treated with UCN-01 were incubated with resin-immobilized MBP-XPA; the bound ERCC1 was determined by immunoblotting of the pull-down complex. Fig. 3A shows that ERCC1 was detectable only in the reaction containing MBP-XPA fusion protein (Lane 1) and not in the reaction containing MBP (Lane 4), suggesting that the ERCC1 detected in the complex was XPA bound. This binding was weakened by UCN-01 treatment (Fig. 3A, Lanes 2 and 3); after normalization for the ERCC1 level in the nuclear extracts as determined by Western blotting, there was a 64% ± 9% reduction of the binding of ERCC1 to XPA in the nuclear extracts from cells treated with 250 nM UCN-01 (Fig. 3A, Lane 3). These results are consistent with the contention that the reduced ERCC1 in the detergent-insoluble fraction of cell lysates...
results from a reduced XPA-ERCC1 interaction. It should be pointed out that in this pull down assay, we did not include nuclear extracts from CDDP-treated cells because we found that the ERCC1 level of the CDDP-treated cells was significantly lower than that of UCN-01-treated cells. Although the exact cause for the lower ERCC1 level is unclear, the binding of ERCC1 to CDDP-damaged DNA may have resulted in the DNA-bound ERCC1 being excluded from the nuclear extracts during extraction. The pull-down assay was also performed using resin-bound MBP-ERCC1 fusion protein to pull down XPA from nuclear extracts. Unlike ERCC1, the amount of XPA bound to MBP-ERCC1 as determined by immunoblotting was about the same when the nuclear extracts from either UCN-01-treated or untreated cells were used in the assay (data not shown). These results imply that, whatever the mechanism may be, the ERCC1 binding domain of XPA was not affected by UCN-01 treatment.

Effect of UCN-01 on Incision. Because ERCC1, when complexed with XPF, is a damage-specific endonuclease, we determined whether UCN-01 affects the incision of the repair process. The incision activity was measured by incorporation of the Klenow enzyme-catalyzed radioactive dAMP incorporation into the excised repair intermediate generated in the modified in vitro repair assay. An UCN-01 dose-dependent decrease of the incision activity was seen in the extracts from UCN-01-treated cells (Fig. 3B). These data are consistent with results in Figs. 2 and 3A indicating that UCN-01 reduced the CDDP-induced subcellular translocation of ERCC1 excinuclease and mediated an attenuation of the XPA-ERCC1 interaction.

Effect of UCN-01 on the Phosphorylation of Repair Proteins. Because UCN-01 is a protein kinase C inhibitor, we speculated that the UCN-01-mediated attenuation of the ERCC1-XPA interaction might result from a posttranslational modification of XPA, ERCC1, and/or other XPA-binding proteins. We examined this possibility using an in vitro phosphorylation assay in which cell extracts were incubated with MBP-ERCC1 or MBP-XPA fusion protein immobilized on resin. The fusion proteins and their binding proteins were pelleted and then subjected to SDS-PAGE, and the protein phosphorylation was visualized by autoradiography of the gel. On the basis of their presumed molecular mass, MBP-ERCC1 and MBP-XPA should migrate in the gel as 80-kDa (Fig. 4A) and 74-kDa (Fig. 4, B and C, left panel) proteins, respectively. However, no band was detected at
these positions in all samples, suggesting that ERCC1 and XPA were not phosphorylated. Fig. 4A shows that CDDP enhanced the band intensity of a 182-kDa ERCC1-bound protein (Lanes 2 and 3); CDDP-induced enhancement, however, was not affected by UCN-01 (Lane 4). These results imply that regardless of whether this protein was an NER-related protein, it was unlikely to be a target of the repair inhibitory effect of UCN-01. Fig. 4B shows that a remarkable band migrating at 52 kDa was detectable in the reaction when MBP-XPA was included, whereas this band was not detected when MBP was substituted for MBP-XPA (comparing Lanes 1 and 2), suggesting that an unidentified protein(s) bound to XPA was phosphorylated. The intensity of this 52-kDa band decreased significantly in the reaction containing cell extracts from UCN-01-treated cells. The effect of UCN-01 was dose dependent; 100 nM produced a 53% reduction, and 250 nM produced an 80% reduction (Fig. 4B, Lanes 3 and 4). The effect of UCN-01 on the 52-kDa protein was also observed in the extracts from cells treated with UCN-01 plus CDDP (Fig. 4B, Lanes 3 and 4, and C, left panel). Interestingly, immunoblotting the MBP-XPA pull-down complex using antibody to the p52 subunit of TFIIH complex detected a band at the same position as this 52-kDa phosphorylated protein (Fig. 4C, right panel).

Although the p52 protein remains to be identified, based on its molecular mass and binding property, we speculated that this phosphorylated, XPA-bound protein could be the p52 subunit of TFIIH. Therefore, the effects of UCN-01 on the TFIIH complex were examined in its p62 and p89 subunits. The results of immunoblotting showed that after normalization for β-actin, UCN-01 did not affect the expression of p62 and p89, but it reduced CDDP-induced relocation of both proteins (Fig. 4D). Such an effect was not seen for RPA34, another protein known to interact with XPA. In fact, UCN-01-enhanced CDDP-induced hyperphosphorylation of RPA34 and the translocation of this species were enhanced by UCN-01 (Fig. 4D). These results also imply that the reduced subcellular translocation induced by UCN-01 does not represent a general phenomenon that occurs with all XPA-binding proteins; rather, the effect may be restricted to TFIIH and ERCC1.

Discussion

The results presented here provide insight into the anticancer action of UCN-01. UCN-01 is known to be a protein kinase C inhibitor and cell cycle checkpoint abrogator; nevertheless, the effects of UCN-01 on DNA repair have not yet been documented. Our data clearly show that UCN-01 inhibited the repair of CDDP-induced DNA damage in an in vitro repair synthesis assay and an in vivo host-cell reactivation assay and demonstrated the inhibitory effect of UCN-01 on the interaction of NER proteins.

In the in vitro repair synthesis as well as repair incision assays, when UCN-01 was included directly in the reaction mixture, the repair activity in the cell extracts was not suppressed (data not shown), implying that UCN-01 may not act by interfering physically with the repair proteins. On the other hand, the repair activity in the extracts from UCN-01-treated cells and the expression of a CDDP-damaged reporter gene in host cells were suppressed by UCN-01 exposure. This
inhibition most likely resulted from UCN-01-mediated regulation of the repair signaling pathway that involves posttranslational modifications of repair proteins. Evidence supporting this theory comes from the finding that although UCN-01 exposure resulted in an elevation of intracellular levels of XPA and ERCC1, the treatment induced a reduction of CDDP-induced translocation of ERCC1 into the detergent-insoluble fraction of cell lysate (Fig. 2C). Moreover, UCN-01 exposure also resulted in a reduced binding of intracellular ERCC1 to the MBP-XPA fusion protein (Fig. 3A), consistent with the contention that UCN-01 inhibits NER through interference with the XPA-ERCC1 interaction. To this end, the paradoxical increase in the basal expression of XPA and ERCC1 in UCN-01-treated cells might arise as a transitional response to the UCN-01-induced increase in the intracellular adduct level, as measured by flameless atomic absorption spectrophotometry (Table 1).

The UCN-01-mediated reduction of the CDDP-induced DNA binding of ERCC1 (Fig. 2C) and the decrease in the binding of intracellular ERCC1 to the MBP-XPA fusion protein (Fig. 3A) were further confirmed by the inhibition of incision activity in the extracts from the cells exposed to UCN-01 (Fig. 3B). It should be pointed out that the incision assay we used measures the overall incision activities of ERCC1/XPF and XPG endonucleases. In this regard, the effect of UCN-01 on the XPF and XPG activities has not yet been determined. Thus far, no study has demonstrated that XPA and ERCC1 are phosphorylated proteins, and in fact, the in vitro phosphorylation assay showed no phosphorylation of these two proteins (Fig. 4, A and B). The lack of phosphorylation has been further confirmed for XPA by the in vivo phosphorylation assay (data not shown) but not for ERCC1 because of the lack of an anti-ERCC1 antibody that would immunoprecipitate the protein. Together, the results argue that the attenuation XPA-ERCC1 interaction was unlikely to have resulted from the posttranslational modification of the protein per se. In this regard, it remains to be determined whether UCN-01 affects the interaction of ERCC1 with XPF and, if does, whether it would in turn interfere the XPA-ERCC1 interaction. Also of note, UCN-01 treatment enhanced CDDP-induced hyperphosphorylation of RPA34 and the translocation of hyperphosphorylated RPA34 into the detergent-insoluble fraction (Fig. 4D). Phosphorylation of RPA34 had no effect on NER activity in in vitro studies (7), and we are at present still unclear what roles overexpression and posttranslational modification of this protein may play.

On the basis of the molecular mass of the dephosphorylated protein and its XPA-binding nature, we speculate that the 52-kDa protein might be the recently cloned p52 subunit of TFIIH (22), although its identity remains to be determined. Immediate attempts to define its identity have met with difficulties because an antibody that would immunoprecipitate the p52 subunit of TFIIH is presently not available. However, UCN-01-treated cells showed a reduced quantity of p62 and p89 subunits of TFIIH in the detergent-insoluble fraction (Fig. 4D), suggesting that UCN-01 inhibited the translocation of TFIIH. These results also support, but do not prove, the notion that the XPA-bound 52-kDa protein detected in the MBP-XPA pull-down assay may represent the p52 subunit of TFIIH.

In conclusion, our results show that UCN-01 inhibited NER, apparently by interfering with the repair signaling pathway rather than by physically interfering with the repair apparatus. To our knowledge, this report is the first to demonstrate that the cell cycle checkpoint abrogator UCN-01 acts as an NER inhibitor. Inhibition of NER by UCN-01 was associated with an UCN-01-mediated attenuation of the XPA-ERCC1 interaction and UCN-01-induced dephosphorylation of an XPA-bound 52-kDa protein, the identity of which remains to be determined.

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References


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