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Prevention of Cisplatin–DNA Adduct Repair and Potentiation of Cisplatin-Induced Apoptosis in Ovarian Carcinoma Cells by Proteasome Inhibitors

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ABSTRACT. Histones H2A and H2B are known to be reversibly post-translationally modified by ubiquitination. We previously observed in cultured tumor cells that proteasome inhibition stabilizes polyubiquitinated proteins, depletes unconjugated ubiquitin, and thereby promotes the deubiquitination of nucleosomal histones in chromatin. Provocative indirect evidence suggests that histone ubiquitination/deubiquitination cycles alter chromatin structure, which may limit accessibility of DNA repair proteins to damaged sites. In the present study, we focused on the relationship between the ubiquitination status of histone H2A, the structure of chromatin, and the efficiency of nucleotide excision repair (NER) of cisplatin–DNA adducts in human ovarian carcinoma cells exposed to the antitumor drug cisplatin. Pretreating cells with the proteasome inhibitor lactacystin (LC) or *N*-acetyl-leucyl-leucyl-norleucinal (ALLnL) induced deubiquitination of ubiquitinated histone H2A (uH2A) and concomitantly promoted chromatin condensation, increased the extent of cisplatin–DNA adducts, and diminished NER-dependent repair of cisplatin–DNA lesions, compared with control cells treated with cisplatin alone. Both proteasome inhibitors also prevented the increase in ERCC-1 mRNA expression that occurs in cells exposed to cisplatin. Cells treated with the combination of ALLnL and cisplatin underwent apoptosis, as indicated by caspase-dependent poly(ADP-ribose) polymerase (PARP) cleavage, more quickly than cells treated with either agent alone. Additionally, the combination of ALLnL and cisplatin potently increased p53 levels in cell lysates and stimulated the binding of p53 to chromatin. Together, these observations suggest that proteasome inhibition may be exploited therapeutically for its potential to sensitize ovarian tumor cells to cisplatin. BIOCHEM PHARMACOL **60**;9:1343–1354, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. proteasome; ubiquitin; nucleosomal histones; cisplatin; ERCC-1; DNA repair

Cisplatin and carboplatin are among the most effective anticancer drugs in the treatment of ovarian carcinoma. It is broadly accepted that the antitumor activity of cisplatin results from the formation of platinum–DNA adducts that strongly interfere with the processing of genomic information [1–3]. Cisplatin–DNA damage—similar to the damage to DNA caused by ultraviolet light or DNA-targeting covalently binding polycyclic aromatic hydrocarbons—is repaired predominantly by the NER‖ machinery. During this process, drug-cross-linked nucleotides as well as adjoining segments containing as many as 40 nucleotides are excised from the damaged DNA strands, and the resulting gaps are patched by resynthesis using the opposite, undamaged DNA strand as a template [4].

The excision nuclease, ERCC-1, makes the initial cut in the DNA strand 5′ to the damaged site [5–7]. The importance of this enzyme is underscored by the observations that cisplatin–DNA adducts cannot be repaired without functional ERCC-1 enzyme [6] and that the absence of ERCC-1 protein is associated with the most severe DNA repair deficit yet discovered [8]. Consistent with this view, upregulation of ERCC-1 mRNA in response to cisplatin exposure correlates with the development by tumor cells of an acquired resistance to this chemotherapeutic agent [9, 10].

In eukaryotic cells, NER involves the cooperative action of more than 25 proteins including several so-called xeroderma pigmentosum (XP) proteins, replication protein A heterotrimers (RPA), and the TFIIH heterocomplex, in addition to the ERCC-1–XPF and –XPG excision nuclease complexes [11, 12]. Although a strict requirement for

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‖ *Abbreviations*: NER, nucleotide excision repair; ALLnL, *N*-acetyl-leucyl-leucyl norleucinal; ERCC-1, excision repair cross-complementation group-1; DAPI, 4,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC, lactacystin; MTT, 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; uH2A, ubiquitinated nucleosomal histone H2A; uH2B, ubiquitinated histone H2B; and XPF, XPG, and XPC, xeroderma pigmentosum groups F, G, and C, respectively.

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protein ubiquitination in NER is yet to be demonstrated rigorously, it is intriguing that the carboxy termini of the human hHR23A and hHR23B Rad23 homologs contain an ~50-amino-acid domain highly homologous to E2 ubiquitin-conjugating enzymes [13, 14]. Intriguingly, hHR23B and hHR23A enzymes form complexes with the XPC DNA damage recognition protein [15]. In yeast, the ubiquitin-conjugating enzyme UBE2I interacts with both Rad51 and Rad52, implying that UBE2I-dependent ubiquitination influences Rad 52-dependent DNA repair [16]. Similarly, in yeast, the Rad6 DNA post-replication repair protein has ubiquitin-conjugating E2 enzyme activity [17, 18], and Rad6 heterodimerizes with Rad18, another component of the DNA repair machinery, which is thought to specifically target Rad6 ubiquitinating activity to sites of DNA damage [18, 19]. The human homologs of Rad6, hHR6A and hHR6B, ubiquitinate histones *in vitro* [20], and these two proteins preferentially localize to transcriptionally active euchromatin [21], which normally is enriched with the monoubiquitinated histones uH2A and uH2B [22, 23]. Most recently, the Rad6-dependent ubiquitination of histone H2B in intact yeast has been implicated in the regulation of mitotic cell proliferation [24]. The Rad6 family of DNA repair enzymes, therefore, may be capable of ubiquitinating nucleosomal histones and specifically altering chromatin structure in regions containing damaged DNA, perhaps as a prerequisite to NER. However, the relationship between the histone ubiquitination status and NER in mammalian cells remains unclear.

The architecture of the nucleosome, with the helical DNA strands wrapped nearly twice around a tight octameric histone core, presents an obvious physical barrier to any repair proteins that must interact with damaged DNA sites. Histone H2A and H2B ubiquitination, the reversible addition of a globular ubiquitin molecule to specific lysine residues located on the flexible histone C-termini, appears to be capable of shifting chromatin from a highly ordered, compact structure to a relaxed conformation [25, 26]. Relaxation of chromatin structure would be expected to make sites of DNA damage in chromatin more accessible to NER machinery. Lending support to this concept, Baxter and Smerdon [27] have shown that nucleosomes transiently unfold during NER in both normal and XPC human cells. Recently, Cai and coworkers [28] cloned a human deubiquitinating enzyme that associates with and possibly regulates the organization of condensing mitotic chromosomes by targeting ubiquitinated nucleosomal histones H2A and H2B. Cells transfected with a mutation-inactivated form of this enzyme stop dividing and undergo apoptosis by an unknown mechanism, illustrating the importance of nucleosomal histone ubiquitination/deubiquitination in cell cycling and survival.

We have reported previously [29] that proteasome inhibitors deplete the intracellular pool of unconjugated ubiquitin and promote the rapid deubiquitination of nucleosomal histones H2A and H2B. This phenomenon is associated with pronounced inhibition of both transcription and replication [29]. In the present study, in cisplatin-resistant A2780/CP70 human ovarian carcinoma cells expressing highly efficient NER activity, we found that proteasome inhibitors increased cisplatin–DNA adduct formation, essentially blocked NER of cisplatin–DNA adducts, prevented cisplatin-dependent ERCC-1 mRNA induction, strongly increased both the cellular level and chromatin association of p53, and potentiated cisplatin-induced apoptosis.

MATERIALS AND METHODS

Tumor Cells and Drug Treatments

A2780/CP70 cisplatin-resistant human ovarian carcinoma cells [30] were propagated in RPMI 1640 medium supplemented with 10% heat-activated fetal bovine serum, 2 mM glutamine, 10 mM HEPES, pH 7.5, under a 5% CO_2 water-saturated atmosphere at 37°. In most experiments, sub-confluent, exponentially growing cells were exposed to ALLnL (100 μM) or LC (10 μM) (stock solutions were dissolved in DMSO) for 4 hr prior to treating cells with cisplatin (*cis*-dichlorodiammineplatinum-II, CDDP or cisPt, Drug Synthesis Branch, NCI, NIH) at 40 or 80 μM for 1 hr. Control cells were treated with 0.1% (v/v) DMSO for equivalent times. Cisplatin was freshly dissolved in PBS to prevent unwanted chemical modification via aquation reactions [31]. The IC_{50} value for cisplatin against A2780/CP70 cells exposed to the drug for 1 hr was 40 μM when determined by the MTT assay 3 days after washing the drug from the cells. The IC_{50} value following 3 days of continuous exposure to cisplatin was 2 μM.

Tumor cells were exposed to cisplatin with or without proteasome inhibitor pretreatment, and the cells were harvested (zero-time or no "recovery") or rinsed once with fresh medium, then re-incubated in the absence or presence of the proteasome inhibitor for an additional 6–12 hr ("recovery"). In other experiments, cells were exposed to ALLnL, cisplatin, or the combination of both agents simultaneously for various times. At the termination of incubations, cells were washed twice in PBS, released from plates by trypsinization, pelleted by centrifugation, and rapidly frozen at −70° until cisplatin–DNA adducts were measured.

For biochemical assays, separate proteasome inhibitor- and cisplatin-treated cells were washed in ice-cold PBS, then lysed on ice in TNESV lysis buffer [50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40 (NP-40), 2 mM EDTA, 100 mM NaCl, 10 mM orthovanadate supplemented with 1 mM phenylmethylsulfonyl fluoride, 20 μg/mL of leupeptin, and 20 μg/mL of aprotinin]. Crude nucleoprotein (mostly NP-40-insoluble chromatin) and supernatant fractions were isolated by centrifugation at 14,000 *g* for 25 min at 4°. The crude chromatin pellets were rinsed once in TNESV, re-centrifuged, then suspended in TNESV by sonication twice at 50 W for 10 sec on ice. In some experiments, intact nuclei were isolated after breaking cells with a Dounce homogenizer. Protein concentrations were measured by the

bicinchoninic acid microplate method, using bovine serum albumin as the standard [32].

Chemicals and Immunoreagents

LC was from Calbiochem. ALLnL and other common laboratory chemicals were obtained from the Sigma Chemical Co. Anti-ubiquitin rabbit polyclonal antibody was purchased from Sigma; horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody was obtained from Amersham Life Science. Rabbit polyclonal anti-PARP antibody was purchased from Upstate Biologicals; anti-p53 mouse monoclonal DO-1 antibody, from Santa Cruz Biotechnology; and the RW017 anti-ERCC-1 antibody was a gift from Dr. Rick Wood, ICRF.

Immunoblot Analysis

After resolving samples by 10 or 16% SDS–PAGE, proteins were electro-transferred by the wet method to Protran™ nitrocellulose membranes (Schleicher & Schuell) overnight (16–20 hr) at 50 V. Membranes used for anti-ubiquitin immunoblots were submerged in deionized water and autoclaved for 30 min to ensure complete denaturation of unconjugated ubiquitin, uH2A, and other ubiquitinated proteins, to enhance antigen recognition by the anti-ubiquitin antibody [33]. After blocking with 5% fat-free dry milk in 10 mM Tris, pH 7.5, 50 mM NaCl, 2.5 mM EDTA buffer, the antigens were immunodetected with appropriate primary antibodies followed by horseradish peroxidase-linked secondary antibodies. Visualization of immune complexes was achieved by chemiluminescence [34], using a luminol-based commercial kit purchased from Pierce. Exposed X-OMAT™ AR films (Eastman Kodak Co.) were developed and scanned (Scanmaker III, Microtek), and the images were processed with a Macintosh™ computer using Adobe Photoshop 3.0 software and quantified using image analysis software (NIH Image).

For unconjugated ubiquitin measurements, tumor cells were rinsed with and then rapidly scraped into ice-cold PBS, boiled for 10 min to inactivate ubiquitin-cleaving isopeptidases, then chilled on ice and centrifuged at 14,000 *g* for 10 min. Aliquots (10–25 μg) of the clarified supernatants were resolved by 16% SDS–PAGE. After electrophoresis, gels were equilibrated at room temperature for 30 min in 65 mM Tris, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, and electro-transferred at 4° overnight at 50 V onto nitrocellulose membranes in 25 mM cyclohexylaminopropane sulfonic acid, pH 10, 20% methanol alkaline transfer buffer [35].

Measurement of Platinated DNA

Tumor cell pellets that had been frozen in tubes at −70° were thawed and lysed in Triton X-100-containing Tris–HCl buffer, and the DNA was isolated by cesium chloride density gradient centrifugation, as previously described [36]. The extracted DNA was dialyzed extensively against distilled water, and then lyophilized after measuring its absorbance at 260 nm. Covalent cisplatin–DNA damage was measured by flameless atomic absorption spectrophotometry, as described in detail previously [37]. Cisplatin–DNA adducts were calculated from a platinum chloride standard curve that was included with each assay, and cisplatin–DNA damage is expressed as femtomoles of platinum per microgram of isolated DNA. The removal of cisplatin adducts from DNA following washout of the drug and re-incubation for 6 or 12 hr was used as a measure of overall cellular NER activity.

Determination of ERCC-1 mRNA and ERCC-1 Protein Levels

Total RNA was isolated from A2780/CP70 cells using a commercial RNA isolation kit (Life Technologies, Inc.). Aliquots of denatured RNA (30 μg) were separated by electrophoresis through 1% agarose–formaldehyde gels, electro-transferred to nylon membranes, prehybridized in Quick-Hyb for 15 min at 68° (Stratagene), and probed in the presence of denatured salmon sperm DNA (Stratagene) with a ^{32}P-labeled anti-ERCC-1 cDNA probe obtained from Dr. A. Sancar (University of North Carolina) or an anti-GAPDH cDNA probe obtained from Dr. M. Olman (University of California), to ensure equal RNA loading, as described in detail elsewhere [38].

To measure ERCC-1 protein levels, cells were treated with cisplatin and ALLnL as in the NER experiments, washed in ice-cold PBS, and scraped directly into 1.0 mL of nuclei isolation buffer [100 mM NaCl, 10 mM Tris, pH 7.5, 5 mM sodium butyrate, 10 mM iodoacetamide, and 0.1% (v/v) NP-40, supplemented with 1 mM phenylmethanesulfonyl fluoride and 10 μg/mL each of aprotinin and leupeptin]. The cell membrane was ruptured with a Dounce homogenizer (20 strokes), and intact nuclei were isolated by centrifugation at 600 *g* for 10 min. The isolated nuclei were washed quickly in cold nuclei isolation buffer, then re-suspended and sonicated in TNESV buffer prior to SDS–PAGE and immunoblotting using the anti-ERCC-1 antibody RW017. Note that ERCC-1 was analyzed in total nuclear protein extracts rather than cell lysates.

Statistical Analysis

Treatment and control groups were evaluated by Student's *t*-test [39] at the 95% confidence level, and significant differences are denoted in the figures by asterisks.

RESULTS

Depletion of Cellular Free Ubiquitin and Promotion of Histone Deubiquitination by ALLnL and LC

We have shown previously that proteasome inhibitors upset the dynamic equilibrium of the histone ubiquitination/deubiquitination cycle by depleting free ubiquitin [29]. This

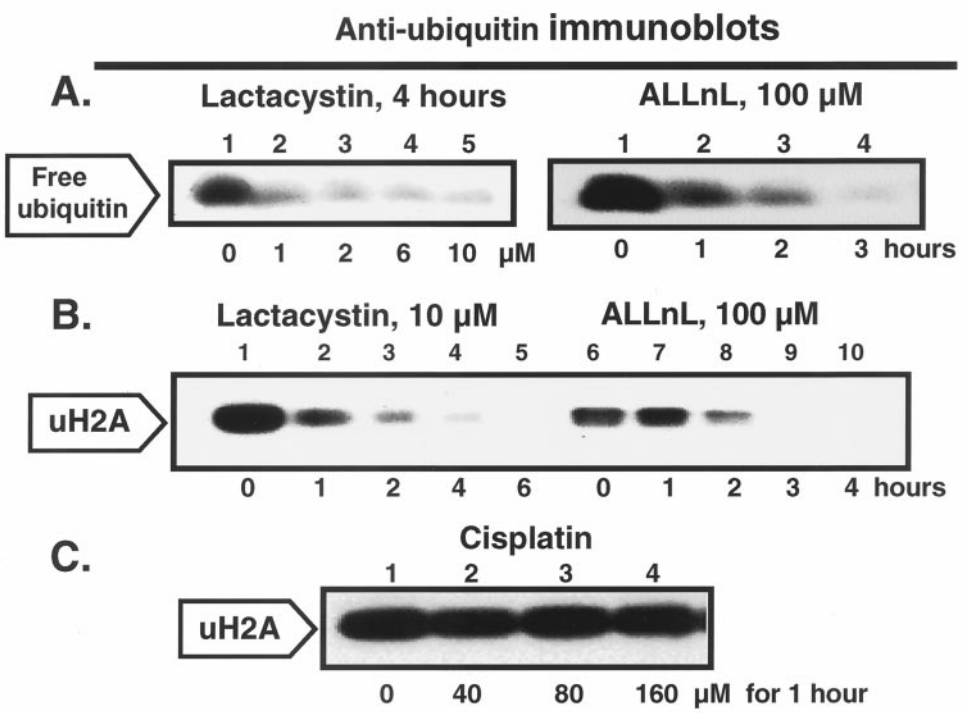

FIG. 1. Depletion of free ubiquitin and deubiquitination of uH2A by proteasome inhibitors. A2780/CP70 human ovarian carcinoma cells were exposed to various concentrations of LC for 4 hr or to ALLnL at 100 μM for up to 3 hr (panel A), and total cellular free ubiquitin was measured by immunoblotting. In panel B, anti-ubiquitin immunoblots of crude chromatin fractions (20 μg) show the time course of uH2A depletion caused by LC and ALLnL. At these concentrations, cisplatin had little or no effect on the cellular level of nucleosomal uH2A (panel C).

is a consequence of the accumulation of highly ubiquitinated proteins that otherwise would be degraded by the proteasome [40–42]. In the current experiments, we determined that both proteasome inhibitors rapidly depleted unconjugated ubiquitin in A2780/CP-70 ovarian tumor cells. LC exposure caused free ubiquitin to decline in a concentration-dependent manner, and 10 μM LC decreased free ubiquitin by 90% within 4 hr (Fig. 1A). In our experience, ALLnL is approximately 1/10 as potent as LC in inhibiting the proteasome; consequently, we exposed cells to 100 μM ALLnL and found that total cellular free ubiquitin was decreased to less than 5% of the control level within 3 hr (Fig. 1A).

Anti-ubiquitin immunoblots of crude chromatin fractions from cells treated with either 100 μM ALLnL or 10 μM LC—concentrations of inhibitors that completely block proteasome activity—revealed significant deubiquitination of uH2A within 2 hr. Following 4–6 hr of exposure to the proteasome inhibitors, uH2A was virtually undetectable (Fig. 1B). Concomitantly with the loss of free ubiquitin and histone deubiquitination, both proteasome inhibitors also promoted the accumulation of large ubiquitinated proteins that were visualized in the range of 200–300 kDa at the top of the anti-ubiquitin immunoblots (data not shown).

Proteasome inhibitor-mediated depletion of uH2A is a consequence of the enzymatic removal of the ubiquitin moiety from histone uH2A rather than the degradation of the histone protein. We have shown previously that ALLnL does not diminish the specific quantity of H2A or alter the profile of any of the nucleosomal core histones in cells [29]. In contrast to the proteasome inhibitors, treating cells with cisplatin for 1 hr caused neither appreciable deubiquitination of uH2A nor accumulation of high molecular weight ubiquitinated proteins, even when cisplatin was used at a concentration as high as 160 μM (Fig. 1C).

Chromatin Condensation and Fragmentation after ALLnL Treatment

Because H2A deubiquitination has been associated with tightening of the nucleosome core and condensation of chromatin structure [25, 43], we evaluated whether ALLnL treatment changed the appearance of chromatin in intact cells. Tumor cells were grown on cover slips, incubated with ALLnL at 100 μM for 12 hr, then exposed to DAPI nuclear stain and photographed using a fluorescence microscope [44]. Visualized by DAPI fluorescence, chromatin in control cells (Fig. 2A) exhibited a homogeneous pattern within regular, ovoid-shaped nuclei. ALLnL-treated cells displayed a heterogeneous chromatin appearance within irregularly shaped nuclei, and in many cells there was conspicuous and extensive chromatin condensation and chromatin fragmentation (Fig. 2B). Importantly, at this time, essentially all of the tumor cells remained viable, as estimated by trypan blue dye exclusion, and morphological

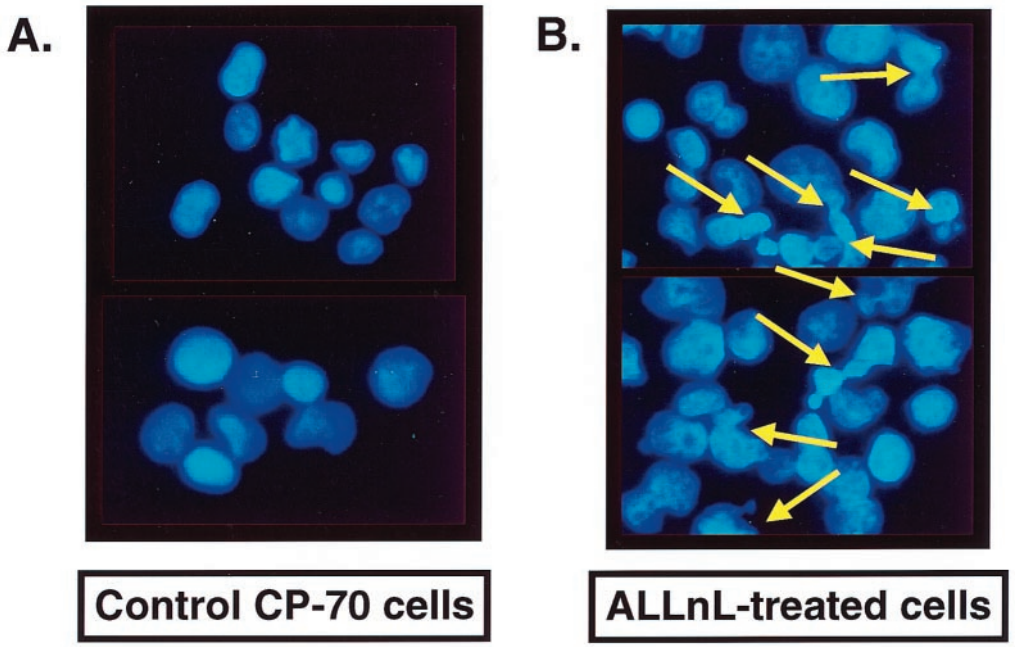

FIG. 2. Effect of proteasome inhibitor on chromatin condensation and fragmentation in A2780/CP70 cells. Tumor cells grown on cover slips were exposed to ALLnL (100 μM) for 12 hr, stained with DAPI, and photographed through a fluorescence microscope. In control cells (panel A), the nuclei appear normal with homogeneous chromatin staining. In cells treated with ALLnL (panel B), the chromatin is noticeably condensed and extensively fragmented (indicated by arrows).

signs of apoptosis (blebbing and cell condensation) were not yet observed.

DNA Platination and Decreased NER-Dependent Removal of Cisplatin from DNA after ALLnL and LC Treatment

We hypothesized that proteasome inhibitor-promoted chromatin condensation might limit the accessibility of DNA-binding proteins, including those involved in NER, to their preferred binding sites. To evaluate the effect of proteasome inhibition on DNA repair activity, we examined the time course of the removal of cisplatin-damaged DNA that had been isolated from cisplatin-treated cells. A2780/CP-70 cells have ample ERCC-1 protein that contributes to their highly efficient DNA repair capability, and it is thought that this phenotype contributes to their remarkable degree of cisplatin resistance [3, 9, 30]. As shown in Fig. 3A, after exposing cells to 80 μM cisplatin for 1 hr, we measured 13 fmol platinum/μg DNA. Interestingly, in cells pretreated with 100 μM ALLnL for 4 hr prior to the cisplatin pulse, the extent of platinum–DNA damage was increased nearly 2-fold to 24 fmol platinum/μg DNA. It is also noteworthy that ALLnL-pretreated tumor cells accumulated approximately 50% more total cisplatin than non-pretreated cells ($4.9 \pm 0.6 \times 10^3$ compared with $3.3 \pm 1 \times 10^3$ fmol platinum/10^6 cells, respectively) after exposure to cisplatin.

We then examined the effect of proteasome inhibitor pretreatment on the efficiency of cisplatin–DNA adduct removal in cells exposed to 80 μM cisplatin for a 1-hr pulse. The cleavage of cisplatin adducts from DNA is a consequence of ERCC-1 activity and constitutes a functional assay for NER. In cells treated with cisplatin alone, the amount of platinated DNA measured immediately after cisplatin exposure declined by approximately 50% during a 6-hr recovery period (Fig. 3A), and 12 hr after cisplatin was removed from cells, the level of cisplatin–DNA adducts was decreased by more than 75% (Fig. 3B). In contrast, in the presence of the reversible proteasome inhibitor ALLnL, the level of cisplatin–DNA damage failed to decrease during the 6-hr recovery period, and in some experiments, DNA platination actually increased slightly after removing cisplatin from the medium, yielding an average of nearly a 4-fold higher level of DNA platination by the end of the 6-hr recovery period compared with cells exposed to cisplatin alone (Fig. 3A).

To verify these observations, we pretreated A2780/CP-70 cells for 4 hr with LC, a very specific and irreversible proteasome inhibitor, and then treated cells with 40 μM cisplatin for 1 hr. In this experiment, cisplatin–DNA damage was measured immediately following cisplatin exposure and after a 12-hr "recovery" period. In cells exposed to cisplatin alone, ~75% of cisplatin–DNA adducts were removed from cisplatin-damaged DNA by 12 hr (16 ± 5 decreased to 4 ± 2 fmol platinum/μg DNA, Fig. 3B). In cells pretreated with LC, the level of DNA platination at time zero was increased by 2-fold (32 ± 8 fmol platinum/μg DNA) compared with controls. Importantly, 12 hr after removing cisplatin, the level of DNA platination in cells exposed to LC plus cisplatin was decreased only slightly

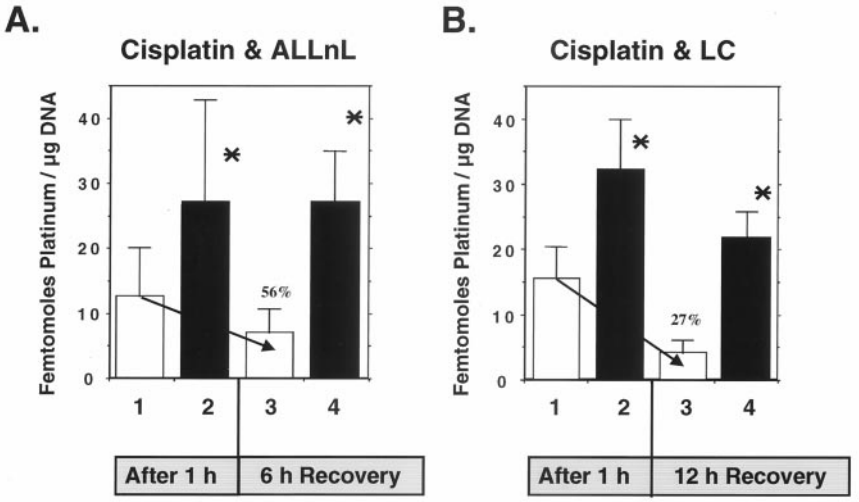

FIG. 3. Proteasome inhibitor-diminished NER of cisplatin–DNA adducts. Tumor cells were treated with ALLnL (100 μM) or LC (10 μM) for 4 hr prior to exposing them to cisplatin (80 μM with ALLnL and 40 μM with LC) for a 1-hr pulse. Cisplatin–DNA adducts were measured immediately after cisplatin treatment or following either a 6-hr (with ALLnL, panel A) or a 12-hr (with LC, panel B) "recovery period." The open bars indicate cells treated with cisplatin alone, and cells treated with cisplatin plus ALLnL (panel A) or cisplatin plus LC (panel B) are indicated by solid bars. Data are expressed as means ± SD. Asterisks indicate differences between values for cisplatin alone and cisplatin plus proteasome inhibitor at $P < 0.05$, as determined by Student's *t*-test. N = 8 for the ALLnL combination; N = 4 for the LC combination. Data were combined from several individual experiments.

(22 ± 4 fmol platinum/μg DNA, Fig. 3B). Thus, after 12 hr of recovery, DNA platination was more than 5-fold greater in cells treated with both LC and cisplatin compared with cells treated with cisplatin alone. These data indicate that proteasome inhibitors strongly interfere with the removal of cisplatin-damaged DNA by the NER enzymatic machinery.

Effects of ALLnL and LC on Cisplatin-Dependent Induction of ERCC-1 mRNA

Cisplatin induces the steady-state level of ERCC-1 mRNA in A2780/CP70 tumor cells, and it has been argued that increased transcription of the *ERCC-1* gene in response to cisplatin exposure contributes significantly to resistance to cisplatin antitumor activity [38]. This premise is supported by data illustrating that agents capable of blocking cisplatin-stimulated up-regulation of ERCC-1 message also sensitize tumor cells to cisplatin [45, 46]. Therefore, we measured ERCC-1 mRNA after treating cells with ALLnL, LC, or cisplatin alone and after the combination of proteasome inhibitor plus cisplatin. We verified the previous observation by Li *et al.* [38] that the cellular level of ERCC-1 mRNA was increased several-fold within 24 hr of exposing cells to cisplatin (Fig. 4, A and B). On the other hand, both LC and ALLnL markedly decreased the steady-state level of ERCC-1 mRNA. Intriguingly, LC, and to a lesser extent ALLnL, abrogated the increase in ERCC-1 mRNA caused by cisplatin (Fig. 4, A and B).

ERCC-1 Protein Levels after Exposure to Cisplatin, ALLnL, and the Combination of Both Agents

Because proteasome inhibitors antagonized the cisplatin-dependent increase in ERCC-1 mRNA, we wanted to determine whether these agents could similarly alter the level of ERCC-1 protein. To this end, we examined the ERCC-1 protein level by immunoblotting nuclear extracts from cells treated with cisplatin, ALLnL, or the combination of the two agents. Exposing cells to 100 μM ALLnL for as long as 6 hr did not change the ERCC-1 protein level; similarly, cisplatin treatment at 80 μM for 1 hr did not alter the amount of ERCC-1 protein (Fig. 5). To our surprise, following cisplatin washout and re-incubation of cells for an additional 6 hr, we detected a moderate, but reproducible, drop in the ERCC-1 protein level, and this was prevented entirely by co-treating cells with ALLnL (Fig. 5). Although a literature search failed to reveal any information, this observation raises the interesting possibility that ERCC-1 may normally be down-regulated by ubiquitination and proteasomal degradation.

ALLnL- and Cisplatin-Induced Cleavage of PARP

Cisplatin kills tumor cells by apoptosis [47], and both ALLnL and LC are known to be weak apoptotic agents [48–50]. To determine whether the combination of cisplatin and ALLnL acts synergistically to induce apoptosis in A2780/CP70 tumor cells, we monitored the caspase-dependent cleavage of 116-kDa PARP to its 85-kDa fragment [51], as a marker of the apoptotic process. Anti-PARP immunoblots revealed that neither ALLnL nor cisplatin alone, at the concentrations used in the DNA repair experiments, caused any PARP cleavage within 24 hr. However, in cells treated simultaneously with the combination of the two agents, PARP was cleaved to the 85-kDa fragment, indicating that the apoptotic caspase cascade had been initiated (Fig. 6A). Although a small amount of

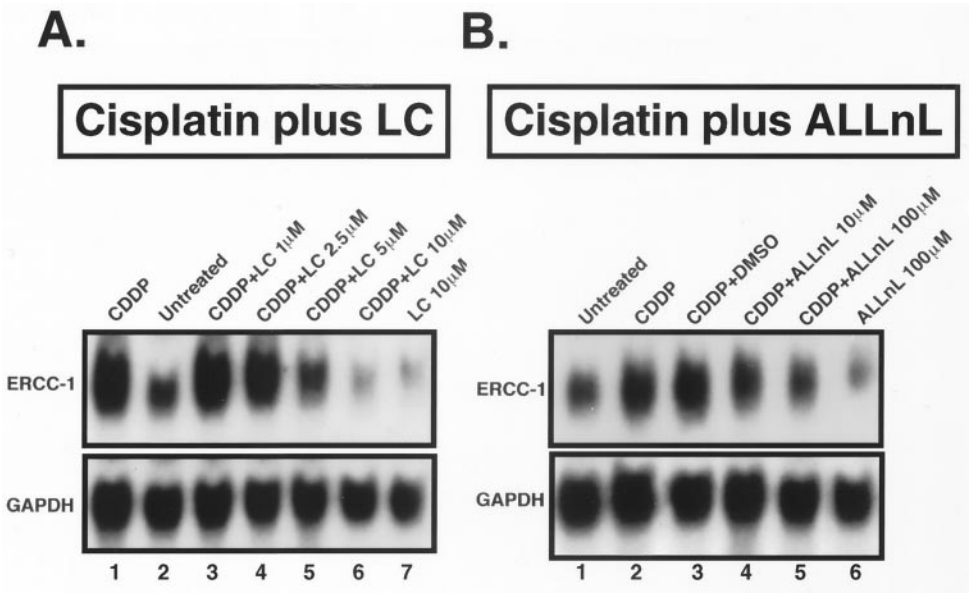

FIG. 4. Effects of proteasome inhibitors and cisplatin on ERCC-1 mRNA. Tumor cells were incubated with 40 μM cisplatin (indicated as CDDP in this figure) for 1 hr or with 40 μM cisplatin for 1 hr following pretreatment with ALLnL (100 μM) or LC (10 μM) for 4 hr. The drug-containing medium was then removed and replaced with fresh medium, and the cells were re-incubated for an additional 24 hr. Northern blot analysis of ERCC-1 mRNA in cells treated either with cisplatin or LC alone, or with cisplatin plus LC are shown in panel A. ERCC-1 mRNA levels from cells treated with either cisplatin or ALLnL alone or cisplatin plus ALLnL are shown in panel B. The blots were subsequently re-hybridized to a radiolabeled GAPDH probe to verify that the amount of RNA loaded onto the gel from each sample was equivalent.

PARP was cleaved in cells exposed to either ALLnL or cisplatin alone for 48 hr, PARP cleavage was always greater in cells exposed to both agents in combination (Fig. 6A). These results are consistent with the hypothesis that the proteasome inhibition-dependent decrease in NER of cisplatin–DNA damage generates a more powerful DNA damage-dependent apoptosis-inducing signal. On the other hand, because ALLnL alone initiated apoptosis, the combination of the proteasome inhibitor with cisplatin may have enhanced apoptosis by independent mechanisms.

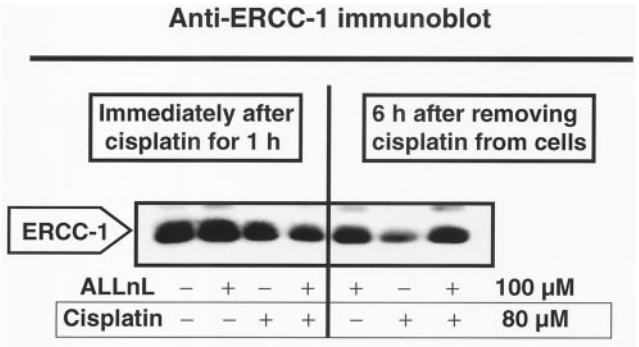

FIG. 5. Effects of cisplatin and ALLnL on the ERCC-1 protein level. Cells were treated with ALLnL at 100 μM for 4 hr followed by a 1-hr pulse with 80 μM cisplatin, and either processed immediately or at the end of a 6-hr "recovery period" in the absence of drug. Intact nuclei were isolated, and lysates were prepared as described in Materials and Methods. Nuclear protein extracts (50 μg) were separated by SDS–PAGE, transferred to nitrocellulose membranes, and probed with an anti-ERCC-1 antibody. The data shown are representative of 3 individual experiments.

Cytosolic and Chromatin-Bound p53 after ALLnL and Cisplatin Treatments

We next examined p53 and uH2A levels in cells treated with ALLnL and cisplatin alone and in combination. Following treatments, the drugs were removed, and the cells were permitted to recover for 6 and 12 hr. In cells treated with 100 μM ALLnL alone or with ALLnL in combination with 80 μM cisplatin, uH2A was depleted nearly completely during the 5-hr treatment period, whereas cisplatin alone caused only a slight decrease in the cellular content of uH2A (Fig. 6B). Following removal of ALLnL, H2A was rapidly re-ubiquitinated, even in those cells exposed to cisplatin. Twelve hours after removing the drugs, uH2A was approximately 50% of the control value in all three treatment groups, illustrating the reversibility of histone deubiquitination. ALLnL and cisplatin, as single agents, increased p53 levels in both lysate (predominantly cytosol) and chromatin fractions, but with markedly different kinetics. p53 initially was elevated strongly by ALLnL and then declined significantly at 6 and 12 hr post-treatment. In contrast, cisplatin alone had no effect on p53 immediately following a 1-hr exposure; however, at 6 and 12 hr following drug washout, p53 was increased several-fold in the drug-treated cells (Fig. 6B). Most interestingly, the combination of both agents caused a rapid and sustained elevation of p53 in both lysate and chromatin fractions, and the high level of p53 persisted for at least 12 hr after drug removal (compare lanes 3, 6, and 9 in Fig. 6B).

These observations prompted us to examine the effects of continuous exposure to ALLnL and cisplatin on cellular

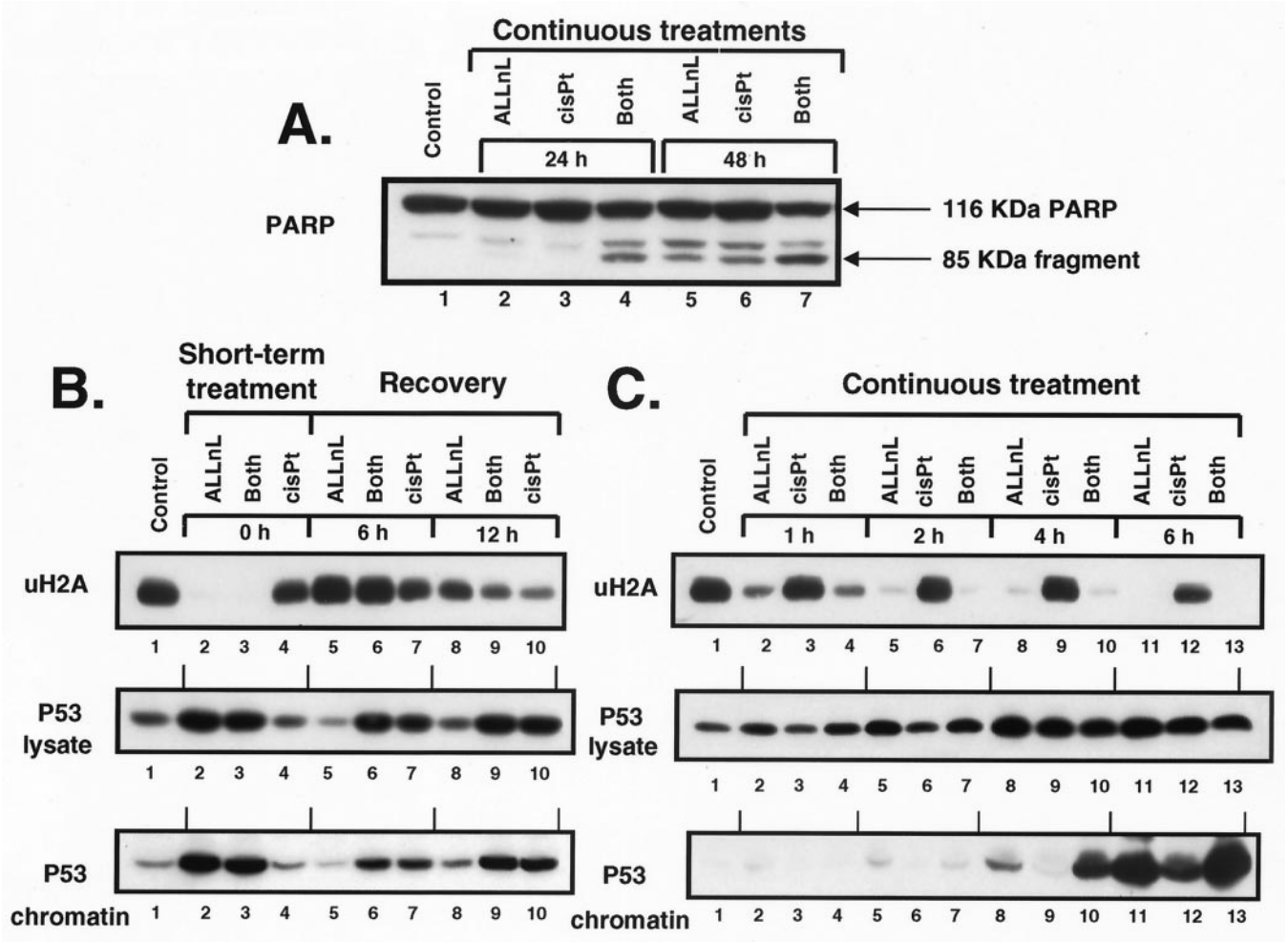

FIG. 6. Potentiation of PARP cleavage by the combination of ALLnL and cisplatin, and stabilization and association of p53 with chromatin. (A) Tumor cells were treated with 33 μM ALLnL or 20 μM cisplatin alone—concentrations that did not cause appreciable cell killing by 48 hr—or with the combination of both agents simultaneously. Chromatin fractions were isolated and separated by SDS–PAGE, transferred to a nitrocellulose membrane, and probed with an anti-PARP antibody that recognizes both the intact 116-kDa enzyme and the 85-kDa caspase-cleaved PARP fragment. A representative experiment is shown. (B) Tumor cells were treated with 100 μM ALLnL and 80 μM cisplatin alone or in combination, as in the NER experiments. uH2A levels in chromatin and p53 levels in both cell lysate and isolated chromatin fractions were examined by immunoblotting immediately following treatments and at 6 and 12 hr after removing the drugs. This experiment was conducted twice. (C) Cells were treated with 100 μM ALLnL and 80 μM cisplatin alone and in combination continuously for 1, 2, 4, and 6 hr, and uH2A and p53 levels were measured by immunoblotting. Note that only a small amount of p53 was associated with chromatin isolated from control cells, but following treatment with ALLnL or cisplatin for 6 hr, considerable p53 was bound to chromatin. This complete experiment was done once, but preliminary experiments were conducted to select appropriate time points.

uH2A and p53 levels. Within 1 hr, ALLnL alone caused substantial deubiquitination of uH2A and elevated p53 in both cell lysate and chromatin fractions by 2 hr (Fig. 6C). Continuous ALLnL treatment for 6 hr eliminated uH2A and increased the p53 level 3- to 4-fold in the lysate fraction compared with controls, with an even greater proportional p53 increase in the chromatin fraction, given that p53 associated with chromatin in control A2780/CP70 cells was low. Cisplatin alone also elevated p53 in the lysate fraction by as early as 2 hr, but had little or no ability to increase p53 associated with chromatin until 6 hr of treatment. In the chromatin fraction, the combination of ALLnL and cisplatin increased the p53 level approximately 5-fold by 4 hr and 10-fold by 6 hr (Fig 6C). This synergistic result is consistent with the hypothesis that prolonged retention of cisplatin-damaged DNA generates signals that initiate recruitment of p53 to chromatin. Alternatively, p53 could associate with chromatin in response to other events, such as stalled replication forks or DNA strand breaks caused by unrepaired cisplatin–DNA damage or by the inhibition of DNA replication caused by ALLnL.

DISCUSSION

ALLnL and LC deplete free ubiquitin from cells by stabilizing polyubiquitinated forms of cytosolic and nuclear proteins that normally would be degraded by the proteasome protease complex [29]. Histone monoubiquitination/

deubiquitination cycling is a tightly regulated enzymatic process, catalyzed by families of nuclear ubiquitin-conjugating and ubiquitin-removing enzymes [43, 52, 53]. The ubiquitin status of nucleosomal histones also depends upon the availability of free ubiquitin in the nucleus [23, 54]. Because the nuclear pool of unconjugated ubiquitin is normally low, depletion of cytosolic, unconjugated ubiquitin rapidly tilts the balance of histone ubiquitination/deubiquitination equilibrium to favor the enzymatic cleavage of ubiquitin [29]. For example, when cells harboring a temperature-sensitive mutated E1 ubiquitin-activating enzyme are shifted to the non-permissive temperature of 39°, histone deubiquitination ensues rapidly [29]. Histone H2A deubiquitination remodels the nucleosomal architecture by tightening the histone octamer core and shifting the chromatin conformation in that vicinity into a condensed, more stable configuration [25, 55]. Proteasome inhibition indirectly promotes nucleosomal histone deubiquitination, and this may induce secondary changes in chromatin structure as a consequence of nucleosomal histone core complex remodeling. In accord with this hypothesis, both LC and ALLnL promoted histone deubiquitination in A2780/CP70 ovarian carcinoma cells and caused abundant, microscopically detectable, DAPI-visualized chromatin condensation. However, whether the chromatin condensation in proteasome inhibitor-treated cells is caused solely and directly by histone deubiquitination remains to be determined.

Such a significant modification of chromatin structure would be expected to interfere with NER of cisplatin-damaged DNA if NER enzymes cannot have access to the cisplatin–DNA damage sites in condensed chromatin. In support of this hypothesis, the amount of platinum bound covalently to DNA in cells exposed to cisplatin for 1 hr was nearly doubled by proteasome inhibitor pretreatment. Perhaps more importantly, there was essentially no loss of DNA–platinum adducts in ALLnL-pretreated cells following a 6-hr "recovery period," and there was a diminished decline in the cisplatin–DNA adducts in LC-treated cells after 12 hr of recovery, results consistent with decreased NER activity. In contrast, in cells treated with cisplatin alone, nearly 75% of the platinum–DNA adducts were excised efficiently within 12 hr after removing cisplatin, verifying that A2780/CP70 ovarian tumor cells possess very active endogenous NER capability. Together, these results suggest a diminished ability of the NER enzymatic machinery to recognize and repair cisplatin-induced DNA damage in proteasome inhibitor-treated cells. Although the increased level of cisplatin–DNA adducts immediately following cisplatin exposure in the proteasome inhibitor-pretreated cells may also have resulted from increased cellular accumulation of cisplatin, the cisplatin–DNA adducts that were formed should have remained susceptible to NER cleavage. Because cisplatin–DNA adducts were not repaired within 6 hr in ALLnL-treated cells, and only moderately repaired 12 hr after LC, other cellular consequences of ALLnL treatment, in addition to increased cisplatin uptake, must have influenced the efficiency of NER.

Although we favor the interpretation that proteasome inhibitor-caused chromatin condensation is primarily responsible for the decreased NER activity in proteasome inhibitor-treated cells, aberrant protein ubiquitination may negatively influence NER in other ways. A possible role for protein ubiquitination in eukaryotic DNA repair is suggested by the function of the Rad6 protein that participates in post-replication repair in yeast [56]. Rad6 is an E2 ubiquitin-conjugating enzyme that heterodimerizes with the Rad18 protein to selectively and efficiently target Rad6 ubiquitinating activity to regions of DNA containing damaged nucleotides [18, 57]. Proteasome mutational analysis indicates that ubiquitination, but not proteolysis, is essential for Rad6-dependent DNA repair activity [19], making it unlikely that Rad6 ubiquitinates proteins that undergo proteasome-dependent degradation. Rad6 has a polyacidic tail that could interact with basic amino acids in histones to facilitate their ubiquitination [58], and unlike most ubiquitinated proteins, monoubiquitinated histones are not proteasome substrates [59], unless they are oxidized [60]. Rad6 is capable of monoubiquitinating histones *in vitro* [21], raising the interesting possibility that Rad6-dependent histone ubiquitination may be linked to DNA repair activity *in vivo*. These findings implicate histones as likely substrates for Rad6, a prediction recently confirmed experimentally *in vivo* by Robzyk and coworkers [24]. Depletion of free ubiquitin pools by proteasome inhibitors would be expected to interfere with Rad6-dependent DNA repair.

It is also possible that proteasome inhibition could interfere with the requisite interactions between ERCC-1 and other components of the NER machinery, such as RPA, XPA, or XPF [61], by stabilizing ubiquitinated forms of these repair proteins. Inappropriately stabilized, ubiquitinated ERCC-1, RPA, XP, or other proteins could, at least in theory, lead to compromised NER if the ubiquitin moiety interferes with their functions. An alternative possibility involves the hHR23A and hHR23B proteins, human homologs of the yeast Rad23 NER protein. Both hHR23A and hHR23B heterodimerize with the XPC protein [15], and these complexes appear to recognize distortions in the DNA caused by various types of damage as a prelude to repair [12]. Rad23 interacts with the proteasome complex through an amino-terminal, ubiquitin-like domain, while the carboxy terminus of Rad23 binds to Rad4, the yeast homolog of human XPC, thereby implicating the proteasome in Rad4 repair function [62]. The human XPC–hHR23B complex is also thought to recognize regions of damaged DNA, although it is not known at this time whether the complex interacts with the proteasome in mammalian cells. If this interaction takes place, then proteasome inhibition could interfere with XPC–hHR23B-dependent DNA repair.

Cisplatin caused a several-fold elevation in ERCC-1 mRNA in A2780/CP70 cells, raising the possibility that induction and/or stabilization of ERCC-1 message contrib-

utes to the efficient repair of cisplatin-damaged DNA in these cells [38]. In this study we have verified that cisplatin increases ERCC-1 message, and we show further that proteasome inhibitors prevented the cisplatin-caused elevation in ERCC-1 mRNA. Interestingly, ERCC-1 mRNA was decreased by proteasome inhibitors in the absence of cisplatin, and this observation is consistent with a previous finding that LC and ALLnL significantly inhibit overall transcription by more than 50% [29]. It is also possible that proteasome inhibition may have decreased ERCC-1 mRNA stability. The decrease in ERCC-1 mRNA appears to have some specificity since the mRNA level of the housekeeping gene *GAPDH* was not altered by either ALLnL or LC (see Fig. 4), and proteasome inhibitors induce, rather than inhibit, the transcription of genes encoding Hsp70 and Hsp90 heat-shock proteins [29, 63, 64]. Although cisplatin increased ERCC-1 mRNA significantly, we failed to observe a corresponding change in ERCC-1 protein, at least within 6 hr following cisplatin exposure. In a previous study [65], cisplatin-dependent induction of ERCC-1 mRNA was followed by an increase in ERCC-1 protein over a protracted 24- to 48-hr time period—up to 4 times longer than the longest time we used to measure NER in this report. Thus, it seems unlikely that proteasome inhibitor-compromised NER of cisplatin–DNA adducts was secondary to effects on ERCC-1.

The combination of ALLnL and cisplatin initiated a strong apoptotic stimulus, compared with either cisplatin or ALLnL alone, based on enhanced caspase-dependent PARP cleavage 24 and 48 hr post-treatment. However, we did not observe significant PARP cleavage at the time points used to evaluate NER of cisplatin–DNA lesions. It may be that cisplatin-caused apoptosis and NER of cisplatin–DNA damage are independent processes, or perhaps the apoptotic caspase cascade initiated by cisplatin in tumor cells is dependent upon the failure of NER. It should be pointed out that both ALLnL and cisplatin alone are capable of causing apoptosis; therefore, the combination of these two agents may have enhanced apoptosis by independent, non-interacting mechanisms.

Chemically caused DNA damage initiates genotoxic stress signals that promote both cytosolic and nuclear accumulation of p53, which transactivates a variety of genes that disrupt cell-cycle progression, permitting repair of damaged DNA to occur prior to the scheduled onset of mitosis [66]. Alternatively, cisplatin-activated p53 is thought to promote apoptosis of cells with irreparably damaged DNA [67, 68]. Although the p53 response to cisplatin in A2780/CP70 cells was delayed, eventually there was a robust increase in p53 protein, which persisted for at least 12 hr after removing cisplatin. In contrast, the increase in p53 protein caused by ALLnL was attenuated within hours of removing the inhibitor. ALLnL and LC stabilize p53 by blocking its ubiquitin-dependent proteasome degradation [69], indirectly stimulating p53-responsive gene transcription and causing either cell-cycle arrest [50, 70] or apoptosis [48, 71]. Based on these observations, we wondered whether the combination of ALLnL and cisplatin would cause a sustained elevation of p53 protein. In cells briefly exposed to both agents, there was a rapid, durable, and apparently synergistic increase in the total cellular content of p53, as well as a marked increase in p53 associated with chromatin. Simultaneous and continuous treatment of cells with both agents elicited an even stronger elevation in p53 as well as a massive association of p53 with chromatin. It is possible that the enhanced binding of p53 to chromatin in ALLnL- and cisplatin-treated cells may be a direct consequence of structural changes in chromatin resulting from ALLnL-mediated nucleosomal histone H2A deubiquitination and that the association of p53 with chromatin may play a pivotal role in the subsequent initiation of apoptosis. This possibility is supported by the observation that PARP cleavage was enhanced in cells treated with ALLnL and cisplatin, compared to cells treated with either agent alone. Although we did not examine p53-dependent gene transcriptional activity in this report, both ALLnL and cisplatin independently induce the expression of p53-dependent genes [66, 71, 72], and one would predict that the combination of both agents would elicit a strong p53-dependent transcriptional response.

In summary, proteasome inhibitors potently antagonized NER of cisplatin-damaged DNA in cisplatin-resistant human ovarian carcinoma cells. Several possible mechanisms involving aberrant or unscheduled ubiquitin utilization, including changes in chromatin fine structure, may mediate the inhibition of NER. Consistent with NER antagonism, the combination of proteasome inhibitor plus cisplatin elevated p53 protein to a higher level than either agent alone and enhanced the apoptotic response of cisplatin-resistant cells. Given that proteasome inhibitors are considered to be a promising new class of anticancer drugs [73], these observations raise the possibility that proteasome inhibition may be exploited therapeutically as an adjuvant in conjunction with cisplatin for treatment of human neoplasia. Such combination chemotherapy may be especially useful in tumors that demonstrate cisplatin resistance resulting from pronounced NER.

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