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Identification of Nuclear Proteins that Interact with Platinum-Modified DNA by Photoaffinity Labeling

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Cisplatin is widely used in the clinical treatment of various malignancies and has especially high potency against testicular cancer. The therapeutic mechanism of cisplatin involves DNA binding, with the formation of 1,2-intrastrand cross-links being the major platinum−DNA reaction. Cisplatin binding to DNA causes a dramatic distortion in the duplex structure, which serves as a recognition signal for repair and other cellular processes.[1] Much effort has focused on isolating and identifying specific proteins that mediate the cellular responses to cisplatin. By screening a human cDNA expression library with cisplatin-modified DNA probes, our laboratory identified a gene that encodes one such structure-specific recognition protein, SSRP1.[2,3] This protein contains a high-mobility group (HMG) domain, a DNA-binding element that recognizes distorted DNA structures including that afforded by Pt 1,2-intrastrand cross-links. This method, however, cannot identify proteins that interact with cisplatin-modified DNA as a consequence of post-translational modification or as part of a multiprotein complex, in which the protein of interest does not directly bind to the damaged DNA.

To probe more generally the binding of proteins to cisplatin−DNA adducts, a series of platinum(II) complexes containing a tethered, photoreactive aryl azide group were prepared, and their ability to form protein photocross-links when bound to DNA was investigated. During the study, DNA modified with cisplatin, initially examined in a control experiment, exhibited higher photocross-linking efficiency when irradiated at 302 nm than did the complexes containing the aryl azide group.[4,5] The mechanism proposed for the formation of the protein cross-links to the cisplatin-modified DNA involves photosubstitution of one platinum-coordinated guanine base by an amino acid in the vicinity of the platinum center. A serious limitation of this method is the requirement of a coordinating and well-positioned protein side chain in near contact with the platinum atom. In our continuing effort to develop a more general photocross-linking approach to identify cellular proteins that are involved in the cisplatin mechanism of action, we describe here the synthesis of cisplatin analogues having a tethered photoreactive benzophenone moiety (Figure 1A). Upon irradiation at 365 nm, a wavelength that does not labilize Pt−guanine bonds, the appended benzophenone group generates a highly reactive species that binds irreversibly to cellular proteins in the vicinity of the platinum-modified DNA probe. With the use of this method, several nuclear proteins were isolated and identified.

A 25 base-pair oligonucleotide duplex containing a single d(GpG) platination site and a biotin moiety at the 3′ end of the bottom strand was modified with PtBPn complexes (Figure 1). The 3′-biotin moiety was introduced to facilitate isolation of the DNA−protein cross-linked adducts by immobilization on a streptavidin-coated solid support. To test the photocross-linking ability of the modified DNA probes, full-length HMGB1 and its HMG-domain constituents were chosen because of their established binding affinity for 1,2-intrastrand platinum−DNA cross-links.[6,7] The

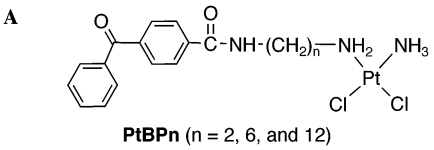

Figure 1. (A) Photoreactive cisplatin analogues with a tethered benzophenone moiety. (B) Oligonucleotide sequence used in the photocross-linking studies. The platinated bases are shown in boldface font.

radiolabeled DNA probes were incubated with the HMGB1 proteins at 0 °C for 30 min to promote the formation of noncovalent DNA−protein binding. Following irradiation with 365 nm light, the reaction mixture was analyzed by gel electrophoresis. The results indicated the formation of the protein−DNA cross-linked adducts (Figure S1). Under the same conditions, a cisplatin-modified DNA probe did not afford any DNA−protein cross-links, consistent with previous work.[4] Of the PtBPn complexes, DNA modified with PtBP6 afforded the highest cross-linking efficiency. Thus, further studies were conducted with the PtBP6-modified DNA.

Since the platinum coordination sphere in PtBPn is unsymmetrical, two orientational isomers can form upon DNA binding, in which the benzophenone is positioned toward either the 3′- or 5′-end of the DNA top strand.[8] In the case of PtBP6, the two isomers can be resolved by ion exchange HPLC (Figure S2). The orientation of the benzophenone group relative to DNA in each isomer has not been determined and is currently under investigation. The two isomers exhibited different photocross-linking efficiencies, however, both with the HMGB1 and with proteins in nuclear extracts (Figure S3). Presumably, there is a more favorable orientation of the benzophenone moiety relative to the DNA-binding proteins in the isomer with higher photocross-linking efficiency.

The isomer with better photocross-linking ability was used to isolate DNA damage-specific binding proteins from a HeLa nuclear cell extract. Preincubation of the modified DNA probe with the HeLa nuclear extract followed by irradiation at 365 nm for 2 h at 0 °C led to the formation of several DNA−protein cross-linked products (Figure 2A). To identify the cross-linked proteins, a large-scale photocross-linking reaction was carried out, and the DNA−protein cross-links were isolated by immobilization onto streptavidin-coated magnetic beads. The cross-linked adducts were then resolved on an SDS-PAGE gel, and three of the proteins were identified by peptide fingerprint mass spectrometric mapping and/or Western blot analysis (Figure 2A,B). The identities of the other cross-linked proteins are currently under investigation.

Among the cross-linked proteins are HMGB1 and HMGB2 (Figure 2), both anticipated because of their well-established binding ability to the 1,2-intrastrand platinum−DNA cross-links.[6,7,9] A protein with ∼120 kDa molecular mass was identified as poly-

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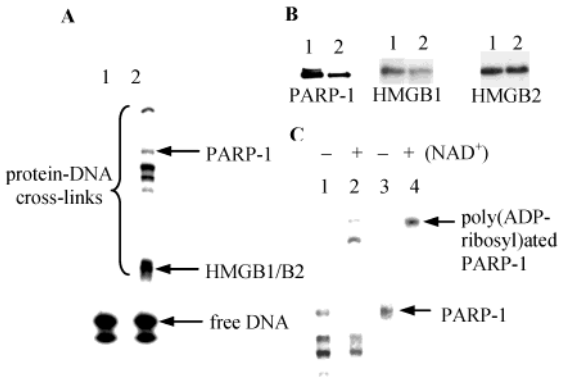

Figure 2. Photocross-linking reactions of PtBP6-modified DNA with HeLa nuclear extract. (A) 10% SDS-PAGE gel demonstrating the formation of several DNA−protein cross-links. Lane 1: radiolabeled DNA probe alone in BSA-containing buffer; lane 2: with HeLa nuclear extract. (B) Western blot analysis showing that HMGB1, HMGB2, and PARP-1 are the cross-linked proteins. Lane 1: photocross-linking reactions; lane 2: HeLa nuclear extract as positive control. (C) The DNA−protein cross-links formed from the photocross-linking reactions in the absence (lanes 1, 3) or in the presence (lanes 2, 4) of NAD^+. Lanes 1 and 2: with HeLa nuclear extract; lanes 3 and 4: with purified PARP-1.

(ADP-ribose)polymerase-1 (PARP-1) by peptide fingerprint mass mapping method. The identity of PARP-1 was confirmed by Western blot analysis (Figure 2B), as well as by a photocross-linking reaction with purified PARP-1 protein. The latter led to the formation of the same DNA−PARP-1 cross-link, as demonstrated by SDS-PAGE (Figure 2C, lane 3). PARP-1 is an abundant nuclear protein which catalyzes the formation and transfer of poly(ADP-ribose) derived from NAD^+ to target proteins including itself in response to DNA damage.[10,11] To test whether the platinated DNA can induce PARP-1 activity, NAD^+ was added to the photocross-linking reaction mixture and incubated for 30 min prior to UV irradiation. As demonstrated in Figure 2C, the presence of NAD^+ in the nuclear extract (lane 2), followed by photocross-linking, afforded two new species with much retarded mobility compared to the cross-linked DNA−PARP-1 adduct observed in the reaction without NAD^+ (lane 1). The slower moving band is assigned to the auto-poly(ADP-ribosyl)ated PARP-1 on the basis of a photocross-linking reaction with purified PARP-1 in the presence of NAD^+ (lane 4).

Although not extensively studied, evidence suggests that PARP-1 has an important function in cellular responses to cisplatin. Treatment with the drug significantly elevates cellular poly(ADP-ribosyl)ation levels, which are mainly due to PARP-1 activity.[12] Furthermore, treatment with PARP-1 inhibitors can increase cell sensitivity to cisplatin.[13,14] A very recent study[15] showed that PARP-1 inhibition can reduce the rate of repair of various types of DNA damage, including cyclobutane pyrimidine dimer formation. Pyrimidine dimers are exclusively repaired by nucleotide excision repair (NER), a major pathway responsible for cisplatin removal. The repair rates, however, were not affected by PARP-1 inhibition in cells deficient in Cockayne syndrome B protein (Csb), an important protein in the transcription-coupled repair (TCR) pathway. This observation suggests a role of PARP-1 in TCR. Both the NER and TCR pathways are implicated for cellular repair of cisplatin−DNA damage.[1,16] Our results providing direct evidence that PARP-1 contacts cisplatin−DNA 1,2-intrastrand cross-links in nuclear cell extracts are thus consistent with the observations in the literature and indicate that poly(ADP-ribosyl)ation activity may be crucial for cellular repair of platinated DNA.

In conclusion, we have discovered a general approach to isolate and identify cellular proteins that interact with platinum-modified DNA based on photoaffinity labeling. With this method, several nuclear proteins have been identified that are likely to be involved in processing cisplatin−DNA adducts in cancer cells.

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Supporting Information Available: A detailed experimental section, a PAGE gel showing the formation of the DNA-HMGB1 cross-links, an HPLC trace of the two orientational isomers of PtBP6-modified 25AGGA, an SDS-PAGE gel showing different photocross-linking efficiencies of the two orientational isomers, and the mass spectrum of the DNA−PARP-1 cross-link (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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