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ARTICLE

HMGB1 bound to Cisplatin-DNA Adducts Undergoes Extensive Acetylation and Phosphorylation in Vivo

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www.rsc.org/Yafeng He^a, Yin Ding^a, Dan Wang^a, Wanjun Zhang^b, Weizhong Chen^a,
Xichun Liu^a, Weijie Qin^b, Xiaohong Qian^b, Hao Chen^{a*} and Zijian Guo^{a*}

Cisplatin, one of the most effective anticancer drugs, is a DNA-damaging agent that induces cell death primarily by apoptosis. For many years, HMGB1 has been known to be a recognition protein for cisplatin-DNA lesions. Here, an application of a biomolecular probe based on peptide-oligonucleotide conjugate is presented as a novel method of investigating this recognition process in vivo. Proteins known to be involved in the recognition of cisplatin-damaged DNA were pulled down and identified, including members of the HMGB family and a number of other proteins. Interestingly, at least 4 subforms of HMGB1 bind to cisplatin-DNA adducts. These proteins were further identified as post-translationally acetylated or phosphorylated forms of HMGB1. These results provide a rich pool of protein candidates whose roles in the mechanism of action of platinum drugs should be explored. These newly discovered molecular components of the DNA damage signalling cascade could serve as novel links between the initial cell responses to DNA damage and the downstream apoptotic or DNA repair pathways.

Introduction

Cisplatin, one of the most widely used anticancer drugs, binds DNA and primarily forms 1,2-d(G*pG*) and 1,2-d(A*pG*) intra-strand cross-links; less frequently, 1,3-d(G*pTpG*) cross-links and inter-strand cross-links are formed.¹ Similar to most clinical anticancer drugs targeting DNA, it is believed that the cisplatin-DNA adducts initiate a series of cellular events, such as blocking DNA replication and gene transcription, triggering diverse signalling pathways. Together, these effects eventually lead to apoptosis or systematic cell death.²⁻⁵ To counteract these effects, DNA repair proteins in the nucleus form a self-defence system against this DNA damage. The removal of certain DNA lesions through DNA repair pathways provides an opportunity for the cancer cell to survive.⁶ Regardless of the type of DNA damage, the recognition of these cisplatin-DNA adducts by certain proteins is the first step in the induction of most downstream cellular events. The direct interaction of Pt-DNA adducts with sensor proteins stimulates the nucleus to generate diverse types of functional machinery that perform standard cell crisis responses. However, the molecular basis for this signalling cascade is still under investigation. Various methods have been used to identify many proteins as Pt-DNA adduct-binding factors.^{7,8} These proteins include DNA damage repair proteins, such as nucleotide excision repair proteins (NER),⁹

mismatch repair proteins (MMR),¹⁰ DNA-dependent protein kinase (DNA-PK), HMG-domain proteins, and several other cellular factors, such as TBP, p53, hUBF, and PARP-1.¹¹⁻¹⁵ Among the most studied of these factors, HMG domain proteins were found to bind preferentially to cisplatin-modified DNA.¹⁶

The HMGB protein, which belongs to the large family of HMG domain proteins, contains three primary members: HMGB1, HMGB2 and HMGB3. HMGB1, which has been considered to be a primary recognition factor of cisplatin-DNA adducts, shows remarkably high affinity to cisplatin-DNA cross-links.¹⁷ As a multifunctional non-chromatin nuclear protein, HMGB1 acts as a molecular chaperon between distorted DNA and various proteins.¹⁸ In contrast to the stabilisation of chromatin by histones, HMGB1 binds with linker DNA between chromatin cores and destabilises compact chromatin DNA. This effect provides access for DNA repair proteins or transcription factors to their cognate DNA site.¹⁹ Through this interaction with HMGB1, proteins that are involved in DNA repair or proapoptotic pathways can respond to cisplatin-induced DNA damage.^{20,21} The phosphorylation of p53 at Ser9 and Ser15²² and the phosphorylation of γ -H2AX, have been considered DNA damage hallmarks of chemotherapy treatment.²³ These biological modifications of key proteins are reduced in HMGB1-deficient cells. This fact suggests that HMGB1 is an

anti-acetylated lysine antibodies (c); schematic illustration of the secondary structures of the HMGB proteins (d).

Table 1. Proteins identified by platinum compound-containing probe affinity isolation from cell extracts

NO.	Protein ID	MW	pI ^[a]	Score ^[b]
1	High-mobility group protein B1	25049	5.45	60
2	High-mobility group protein B2	24190	7.94	95
3	High-mobility group protein B3	22,980	8.48	43 ^[c]
4	High-mobility group protein B1	25049	5.45	62
5	High-mobility group protein B1	25049	5.45	109
6	High-mobility group protein B1	25049	5.45	94

[a] Theoretical pI from ProMoST

[b] Mascot online server PMF score

[c] HMGB3 is identified through its unique peptide fragment MS/MS ions.

Surprisingly, most of the HMGB proteins that bound to cisplatin-DNA adducts are post-translationally modified (PTM) forms, including HMGB1, HMGB2 and HMGB3. Using the isoelectric focusing technique, at least 4 HMGB1 spots (Figure 2b) and 2 HMGB2 spots (Figure S7) with essentially the same molecular weights but different pIs (isoelectrical point) were identified using peptide mass fingerprinting. The apparent pI of HMGB3 is approximately 4.8, which is very different from its canonical pI of 8.48 (Figure 2b). These results were verified through 2-DE followed by western blotting (2D-WB). The theoretical pIs predicted by the protein modification screening tool (ProMoST)³¹ for canonical HMGB1/2 are 5.45/7.94, respectively. Both of these proteins that were captured and identified with the described probe exhibit multiple types of modification, and the protein spot on the acidic end of 2-DE strip shows a remarkable pI shift. These results suggest the presence of different forms of HMGB protein that vary by their global charge distribution. As HMGB1, HMGB2 and HMGB3 exhibit over 80% amino acid sequence identity and possess the same DNA binding domains,¹⁹ (Figure 2d), further detailed studies focused on forms of HMGB1 with diverse pI isoforms and important biological functions. According to a previous study, HMGB1 might be acetylated *in vivo*. Therefore, the HMGB1 protein pulled down with our probe was immunoblotted with a specific anti-acetylated lysine antibody (Figure 2c). The result clearly shows that at least a portion of the HMGB1 that binds to the Pt-DNA adduct is acetylated in the cell.

Using HMGB1 as an internal marker, the pull-down results indicate that this protein could bind with cisplatin-damaged DNA at a DNA concentration of 50 nM (Figure 3b). This binding is stronger than the *in vitro* measured binding affinity ($K_d = 120$ nM) of HMGB1 for cisplatin-DNA cross-links.³² To survey the natural distribution of PTM isoforms of HMGB1 *in vivo*, an anti-HMGB1 antibody was used to immunoprecipitate

(IP) HMGB1 in the same cell extract used in the capturing experiments with the probe. The IP proteins were resolved with 2-DE and then immunoblotted with the same antibody. As shown in Figure 3e, there is a unique distribution of HMGB1 PTM isoforms in the cell, and isoforms A and B can be recognised by 2 different methods with similar binding affinities. However, isoforms C and D exhibit higher binding affinity to the probe containing the cisplatin lesion than to the traditional antibody. The protein spots pattern of HMGB1 in Figure 3d is consistent with the pattern in Figure 2b. HMGB1 subform D exhibits a highly significant pI difference from subforms A-C, suggesting its unique modification status. In addition, the 4 subforms captured with our probe are found in markedly different abundances. These variable abundances might suggest their different binding affinity to cisplatin-DNA cross-links.

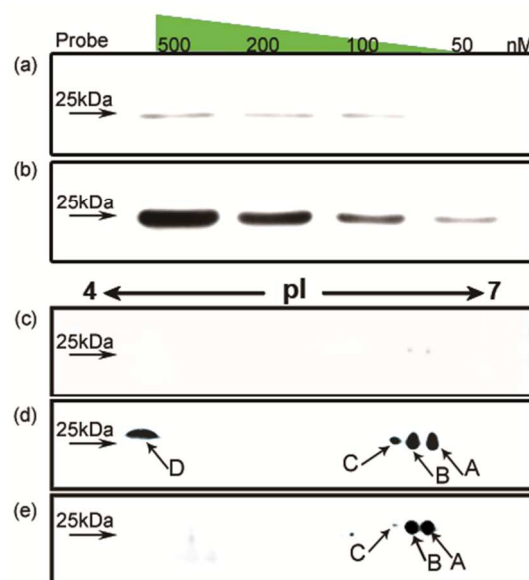


Figure 3. Investigation of HMGB1 isoforms in the cell. HMGB1 is captured from SKOV3 cell extracts by probes with (b) or without (a) cisplatin cross-links; the concentration of the probe is varied from 50 nM to 500 nM. The analysis by 2D-WB shows HMGB1 isoforms trapped by the cisplatin probe (d) or immunoprecipitated with anti-HMGB1 antibody (e); (c) probe without cisplatin cross-link acts as a control.

Mapping the acetylation and phosphorylation sites of HMGB1 subforms

It has been reported in protein translocation studies that several HMGB1 subforms in the calf thymus or activated human monocytes correspond to different acetylation statuses.³³ 2 isoforms of HMGB1 that possess a small number of acetylation sites were reported more than three decades ago.³⁴ Here, we show that there are at least 4 post-translationally modified forms of HMGB1 that recognise cisplatin-DNA 1,2-(GpG) cross-links. To determine the precise modification sites, each of these isoforms was separated on a 2-DE gel and fully characterised using high-resolution LC-MS/MS. As shown in Figure 4, a total of 23 modification sites were identified (Table S2, Figure S8). Surprisingly, both of the HMG boxes of

HMGB1, which are known DNA binding domains, are hyper-acetylated. In addition, 4 acetylated lysines are located at the basic linker sequence between the 2 HMG boxes, and 3 acetylated lysines are present at the linker sequence between HMG box B and the acidic tail. However, no modifications were detected at the acidic tail, indicating its unique conserved property in this protein. In total, 16 acetylated lysine sites were detected in both the A and B isoform of HMGB1. For the C and D isoforms, 3 phosphorylation sites each were observed. For isoform C, three phosphorylated serines are crowded within HMG box A. Isoform D has one phosphorylated serine in each HMG box and contains a phosphorylated threonine in HMG box B. These 2 phosphorylation patterns of HMGB1 have never been detected before. Considering the low abundance of phosphorylated peptide in the mass spectrum data, we believe there could be more phosphorylated sites than were detected in isoform D, resulting in its significant pI range.

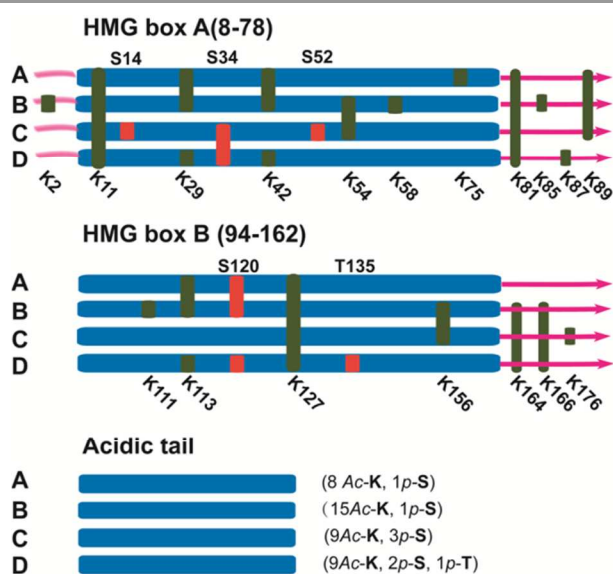


Figure 4. Illustration of the post-translational modification sites of the HMGB1 isoforms. Acetylated lysine sites are marked with K (green ribbon), and phosphorylated serine or threonine sites are abbreviated as S or T (red ribbon).

Discussion

Post-translational modifications of proteins are among the most important cellular events involved in signal transduction. As the most commonly reversible modification of proteins *in vivo*, acetylation and phosphorylation at distinct amino acids alter both the local molecular structure and the charge distribution of the parental protein; further changes to the entire protein conformation then regulate interactions with different binding partners.³⁵ The study of HMGB1 acetylation has primarily focused on lys2. *In vitro* experiments have demonstrated that acetylated lys2 enhances HMGB1 binding affinity to its distorted DNA substrates (e.g., UV- and cisplatin-damaged DNA, 4-way junctions).³⁶ Nevertheless, it is difficult to explain how acetylated lysines affect HMGB1 binding affinity to cisplatin-DNA lesions without further insight into this protein.

The newly identified acetylation sites (Table S2) could provide a wide variety of patterns that regulate HMGB1 interactions with DNA or companion proteins.^{36, 37} For example, as one of the most important proteins interacting with HMGB1, p53 is believed to control several key biological processes along with HMGB1. Our preliminary Co-IP assay results (Figure S9) indicate that p53 protein indeed interacts directly with acetylated HMGB1, and p53 phosphorylated at ser20 could be recognized but not that phosphorylated on ser15. This distinction suggests the acetylation pattern of HMGB1 discussed above might play critical role in p53 pathway which is related to DNA damage response.³⁸ Even more importantly, 2 phosphorylated HMGB1 subforms have also been identified. The phosphorylation of certain amino acids alters the local charge much more than acetylation, an effect that directly results in the large pI shift of the protein. It is interesting that all of the phosphorylation sites are located in the 2 HMG boxes, which are assumed to have different functions based on recent evidence. Box A may interact with other transcription factors as a protein chaperone, and box B may act a DNA-bending factor.^{18, 25} The interplay of the HMG box and the acidic tail is believed to modulate the binding affinity between HMGB1 and its substrate.^{39, 40} The HMG box modification sites revealed here may participate in the interaction with the acidic tail. Specific acetylation and phosphorylation events neutralise the basic HMG boxes and their short basic linker. This change in the local charge distribution disrupts the mask effect of the acidic tail, reinforcing DNA binding and bending abilities. The possible biological relevance of this discovery may be rooted in the local conformational change that occurs after phosphorylation. Increased binding and bending ability of phosphorylated HMGB1 could facilitate the exposure of damaged DNA to repair proteins or other signal factors. It could be hypothesised that HMGB1 both (i) enhances the interaction between cisplatin-DNA lesions and other repair proteins and (ii) recognises DNA damage sites and recruits repair proteins through protein-protein interactions. In both situations, the binding strength can be subtly modulated with posttranslational modifications, as discussed above. Nevertheless, the cellular events that occur after the recognition of damage sites are unclear; studies of these downstream molecular mechanisms are required.

Conclusions

In summary, we have developed a systematic method for the discovery of proteins that are correlated with cisplatin pharmacology. Interestingly, we find that certain HMGB1 subforms within the cell are post-translationally modified. First, unexpected hyper-acetylation is detected on the 4 HMGB1 isoforms. Furthermore, the PTM isoforms C and D, which are differentially phosphorylated, exhibit fairly high-affinity binding to cisplatin-DNA adducts. These results provide an in-depth view of the cisplatin-DNA-protein interaction *in vivo*. Furthermore, these findings may provide new clues towards the

improvement of existing chemotherapies in terms of efficiency and overcoming resistance.

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Notes and references

^a State Key Laboratory of Coordination Chemistry, State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, No. 22 Hankou Road, Nanjing, 210093 (P. R. China); E-mail: chen hao@nju.edu.cn; zguo@nju.edu.cn.

^b National Center for Protein Sciences Beijing, State Key Laboratory of Proteomics, Beijing Proteome Research Center, Institute of Radiation Medicine, 33 Life Science Park Road, Changping District, Beijing, 102206 (P. R. China)

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